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AN EXPLANATION
TO SUBSCRIBERS

Readers of this and subsequent issues of this Journal may observe that the pages are somewhat altered in appearance. What is known as the format has been changed. The Journal is now printed on type known as 10-point on a 12-point body, and the type-page is now 29 picas wide and 47 picas long. (A pica is one-sixth of an inch; a point is one-seventy-second of an inch.)

The reason for the change is this: An acute shortage of paper is about to develop. Paper will be increasingly hard to get and it may come to rationing. In the latter case, those who have already made some effort to save paper will doubtless receive more consideration than those who have not.

The new format will permit more words to be printed on fewer pages, without loss of legibility, and without curtailment of the amount of material published. The same amount will be printed on fewer pages—that is all. Issues will be thinner but- to repeat in order to emphasize—just as much material will be published in each volume as has hitherto been published.

In brief, then, the change

1. Tends to forestall a threatened break in the continuity of publication of scientific material, or a curtailment of the amount published;

2. It is a way to assist in the general problem of national defense;

3. It tends to forestall, delay, or minimize a possible rise in price. Labor and materials are going up. Saving paper partly neutralizes the effect.

Finally, some pages in this issue may be printed in the old format. This is merely because these pages happened to be already set in type when the change was determined upon.

STUDIES ON KETOSIS

XX. THE EFFECT OF GLYCOGEN ON THE OXIDATION OF BUTYRIC ACID BY RAT LIVER SLICES*

By BLANCHE G. BOBBITT AND HARRY J. DEUEL, JR.

(From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles)

The mechanism whereby the metabolism of carbohydrate prevents ketosis has been a controversial subject for many years. Two schools of thought have developed: the one proposing antiketogenesis, the other, ketolysis. The proponents of antiketogenesis believe that carbohydrate exerts a fat-sparing action, hence is metabolized in preference to fat. Adherents to the theory of ketolysis affirm that carbohydrate prevents ketosis by combining with the ketone bodies to form compounds which are then easily oxidized.

Probably the greatest support for the catalytic rôle of glucose is found in the *in vitro* results of Shaffer (1). Shapiro (2) showed that only glucose formers can lessen ketonuria when ketogenic acids are administered, while a similar relationship was also noted in studies with various amino acids by Butts, Dunn, and Hallman (3) and Butts, Blunden, and Dunn (4).

In a study of the comparative action of the fat-sparing substances, glucose and alcohol, on exogenous and endogenous ketonuria, Deuel *et al.* (5) demonstrated that only glucose caused a decrease in acetonuria. Glucose brought about an almost complete abolition of the endogenous ketonuria in fasting rats previously fed a high fat, low protein diet, while ethyl alcohol was entirely ineffective. Calculations showed that the extent of fat oxidation was practically identical in the fasting rats and in those receiving sufficient glucose to overcome the endogenous ketonuria. Also, the amount of glucose which was ketolytic amounted to only a little more than 1 per cent of the total fat oxidation of the day.

On the other hand, Mirsky, Nelson, and Grayman (6) were unable to demonstrate any greater speed of disappearance of injected *dl-β*-hydroxybutyrate by analysis of the tissues of fasted rats than with well fed animals.

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They concluded that the action of glucose was antiketogenic rather than ketolytic. However, Deuel, Hallman, Greeley, Butts, and Halliday (7) found that when l-β-hydroxybutyrate was employed glucose increased the rate of utilization to a considerable extent.

Another possible approach to this problem is by the use of the tissue slice method of Warburg (8). Quastel and Wheatley (9) have demonstrated that acetoacetic acid originates to the greatest extent when the even chained fatty acids are oxidized by liver slices, although small quantities were formed with the odd chain acids. The possible relationship of carbohydrate metabolism to the disappearance of the ketone bodies is indicated by the decreased quantities of acetone bodies which were found when glycogen was also present as a substrate. Glucose did not behave in a similar manner, however, nor were these workers able to repeat these observations on the effect of glycogen a year later (10). The possible application of the tissue slice technique is also indicated in the experiments of Cohen and Stark (11) who demonstrated that a low liver glycogen in rats was accompanied by a greater concentration in ketone bodies both in the absence and presence of butyrate as a substrate. In neither of these studies is it certain that the reduced ketone body content is the result of an increased oxidation or a preferential oxidation of the carbohydrate.

In the present investigation it was desired to determine whether the decreased ketone bodies found when liver slices are present in a butyrate medium containing glycogen as contrasted with the level when this polysaccharide is absent are to be ascribed to a suppression in their formation, which would support the antiketogenesis theory, or to an acceleration in their disappearance, which would support the idea of ketolysis. By the determination of the disappearance of butyrate as well as acetone bodies, it is believed that an answer to this problem has been obtained.

Procedure

The experimental animals used were adult male albino rats, 4 months or more of age, either well fed or fasted for 24, 48, or 72 hours. Before the livers were removed for sectioning, the animals were anesthetized with sodium amytal and bled from the throat.

For the Warburg manometric method a bank of seven constant volume type of respirometers was available. The water bath in which the manometer vessels were shaken was maintained at $39° \pm 0.02°$. Lead perchlorate prepared according to Krebs (12) was used as the manometer fluid.

For sectioning the liver tissue, as an alternative to the usual straight razor a handle was fashioned of spring metal which could be fitted into the slot of an ordinary double edged safety razor blade by means of grooves. Rests or finger grips were attached at either end of the handle so that effort

of approximately the same value was exerted from either hand while sectioning.

Tissue slices not exceeding 20 mg. of dry weight were used. The slices prepared from rat liver immediately after excision of the liver were bathed in 0.9 per cent sodium chloride and then immersed in the manometer vessels. Usually three slices were used in each vessel, so that the average thickness, 0.2 to 0.4 mm., in different vessels would be approximately the same.

The medium in which the slices were immersed consisted of the following: Salt mixture[1] 0.3 ml., phosphate buffer[2] 0.6 ml., sodium chloride, 0.16 M, 2.1 ml. When additional metabolites were added, as sodium butyrate[3] or glycogen,[4] they replaced a similar volume of sodium chloride solution. Filter paper with 0.2 ml. of 2 N sodium hydroxide was inserted in the inner cup of each manometer vessel to absorb carbon dioxide evolved during respiration of the tissues. The gas phase was 95 per cent oxygen and 5 per cent carbon dioxide. After a 2 hour period of respiration, the tissue slices were removed, washed, dried at 105°, and weighed to constant weight. The pH of each vessel was checked. The filter paper absorbers were removed from the vessels, residual alkali neutralized, and the cups dried out. Acetoacetic acid was then determined either manometrically by decomposition with aniline according to Quastel and Wheatley (9) or by the micro acetone method of Edson (13) in which Rupp's (14) iodometric titration for mercury was employed.

The various quotients used to express the results of the respiration experiments are as follows: Q_{Ac} = c.mm. of carbon dioxide equivalent to acetoacetic acid formed per mg. of dry tissue per hour; Q_{BOH} = c.mm. of carbon dioxide equivalent to β-hydroxybutyric acid formed per mg. of dry tissue per hour; Q_{Ket} = sum of Q_{Ac} and Q_{BOH}.

For the determination of residual butyric acid, a distillation procedure was developed for use on the filtrate obtained after precipitation of the acetone-mercury precipitates due to acetoacetic and to β-hydroxybutyric acids. Such filtrates were made up to 250 ml., and 185 ml. were distilled in an all-glass distillation apparatus consisting of a Kjeldahl flask, connecting tube with Hopkins' trap, and a condenser fitted into a filter flask. The distillate was immediately titrated with 0.01 N sodium hydroxide, with phenolphthalein as indicator. The validity of the method was tested

[1] The stock solution was prepared as follows: 360 ml. of 0.16 M KCl, 90 ml. of 0.107 M CaCl₂, 72 ml. of 0.107 M MgCl₂.

[2] Buffer of 0.10 M NaH₂PO₄ adjusted to pH 7.3 with NaOH.

[3] Sodium butyrate 0.16 M.

[4] Glycogen, 0.4 or 0.8 gm., dissolved in 25 ml. of 0.16 M NaCl. Identical effects were obtained with dog liver and abalone glycogens (prepared by Dr. M. G. Morehouse) and with human liver glycogen prepared by the author.

by recovering known amounts of butyric acid from filtrates remaining after precipitation of the acetone bodies from solutions prepared as for respiration experiments but to which no tissues were added. A recovery factor of 93.1 per cent was found.

Results

A study on the effect of fasting on acetoacetic acid content with and without butyrate substrate is summarized in Table I. Because consistently higher values of acetoacetic acid quotients were obtained with the

TABLE I

Effect of Fasting with and without Butyrate on Q_{Ac} of Rat Liver Slices

Group No.	No butyrate in medium				0.01 M butyrate			
	Period of fasting							
	0 hr.	24 hrs.	48 hrs.	72 hrs.	0 hr.	24 hrs.	48 hrs.	72 hrs.
1	1.1	2.0	2.6	2.2	4.3	5.6	5.2	5.6
	1 1	2 0	2.3	2 0	4.2	5.3		6.2
				2.2				6.1
2	1.2	0	1.5	1 8	3.3		4.5	3.4
	1.1		1.8	1.6	4.5			2.7
				1.3				4.4
3	1.1		3.4		3.0		7.8	
	1.1		4.0		2.8		6.7	
			3.5					
			2.1					
4			2.1				5.3	
			2.1				6.0	
			2.0				6.2	
5			2.1				{5.9 / 5.4	
6			2.2				{6.6 / 6.7	
7			2.7				{7.2 / 6.4	

animals fasted 48 hours, such nutritional states were employed in the remaining experiments.

Determinations for Q_{O_2} were made in all tests and in all cases the tissues were respiring in a normal manner. However, since the possibility exists that the CO_2 in the gas mixture used for the preliminary flushing of the system may have partially exhausted the alkali in the flask, these are to be considered as minimum values. In the series reported in Table IV, they averaged −7.69 for the experiments with no addition to substrate, −8.40 for those to which glycogen had been added, −12.00 to those where

TABLE II

Effect of Glycogen on Acetoacetate Quotient of Liver Slices of Unfasted Rats and of Fasted Rats with and without Butyrate

Group No.	Unfasted		Fasted 48 hrs.			
	No glycogen	1 per cent glycogen	No glycogen	1 per cent glycogen	0.01 M butyrate alone	0.01 M butyrate + 1 per cent glycogen
1	2.0	0	2.8	0.1		5.9
		0		2.8		
				0.8		
2	1.8	1.6	3.0	2.4		5.1
	1.2	1.5		2.8		4.6
3	1.3	1.3	1.5	1.4		
	1.1	0.5 ·	1.1	0		
		0.3		0.2		
		1.2		0.6		
4	1.3	0.2	3.0	0.2		
	1.1	0.2	2.6	1.0		
		0.6		1.5		
		1.7		0		
5	1.9	1.8	1.5	0.3		
	1.8	1.8	1.4	0.4		
	2.0	1.8	1.4	1.1		
6		1.6	2.1	0.5		
		1.5				
7			2.1	1.3	5.9	3.9
					5.4	4.4
8			2 2	1 7	6 6	5.0
					6.7	5.8
9			2.7	2.0	7.2	4.9
					6.4	5.4

TABLE III

Typical Experiment Showing Effect of Glycogen on Acetoacetate, β-Hydroxybutyrate, and on Butyrate Concentration of Liver Slices of Fasting Rat

Manometer No.	1	2	3	4*	5	6	7
Salt mixture (K, Ca, Mg), ml.	0.3	0.3	0.3	0.3	0 3	0.3	0.3
Phosphate buffer, ml.	0.6	0 6	0.6	0.6	0.6	0.6	0.6
Glycogen, ml.			1 9		1 9	1.9	
Butyrate, ml.						0.2	0.2
NaCl, ml..	2.1	2.1	0.2	2.1	0.2		1.9
NaOH in inner cup, ml.	0.2	0 2	0.2	0.2	0.2	0.2	0.2
Liver slices	3	3	3	0	3	3	3
Tissue weight, mg.	12.0	7.9	8 8		8 0	8.7	8.2
Q_{Ac}	0.83	0.96	1.01		1.49	4.05	5.40
Q_{BOH}	1.71	2.45	1.45		0.97	2.84	3.41
Q_{Ket}	2.54	3.41	2.46		2.46	6.89	8.81
Residual butyric, ml. 0.01 N NaOH.	0.55	0.56	0.54		0.57	2.38	2.54
Residual butyric corrected, ml.						1.82	1.98

* Manometer 4 was used as a barometer blank

TABLE IV

Summary Table Showing Effect of Glycogen on Acetoacetate, β-Hydroxybutyrate, and on Butyrate Concentration of Liver Slices of Fasting Rat

Component determined	Rat liver slices with			
	No substrate	Glycogen	Butyrate	Butyrate and glycogen
Q_{Ac}. Maximum	3.31	1.92	6.67	5.27
Minimum	0.75	0.65	2.38	2.04
Average	1.26	1.18	4.89	3.82
Q_{BOH}. Maximum	2.45	1.88	3.41	2.84
Minimum	0.95	0.38	1.36	1.07
Average	1.52	0.97	2.19	1.87
Q_{Ket}. Maximum	5.37	3.80	9.32	8.05
Minimum	1 77	1.32	3.74	3.15
Average	2.79	2.14	7.09	5.68
Q_{Ket}. Corrected for basal; average			4.30	3.54
Butyrate added, γ			2818	2818
Butyrate recovered*				
Maximum, γ			2405	2322
Minimum, γ			2086	1939
Average, γ			2257	2118
Butyrate disappeared;† average, γ			561	700
Acetone bodies recovered per 10 mg. dry tissue as butyrate; average, γ			338	278
Butyrate disappeared per 10 mg. dry tissue; average, γ			496	594

* The butyrate recovered was calculated by adding the total acetoacetate and β-hydroxybutyrate (corrected for endogenous formation) plus butyrate present as such (corrected for 93 per cent recovery). The formulas for calculation in individual experiments are as follows:

Acetoacetate—

(Q_{Ac} obtained minus Q_{Ac} of control) × mg. tissue × 2 (hrs.) = acetoacetate present as microliters CO_2 (1)

*β-Hydroxybutyrate—*Microliters CO_2 (2) obtained as above

To convert to micrograms of butyrate

(microliters CO_2 (1) + microliters CO_2 (2)) × $\dfrac{88,000,000}{22,400,000}$ = micrograms ketone bodies recovered (as butyrate)

Butyrate as such—

(Ml. 0.01 N NaOH obtained minus ml. 0.01 N NaOH of control) corrected for 93% recovery

Corrected titration ≈ 880 γ butyric acid per ml. 0.01 N NaOH

† Difference between butyrate added and butyrate recovered (as acetoacetate, β-hydroxybutyrate, and butyric added).

butyrate was present, and −11.23 for the flasks containing both the glycogen and butyrate.

The effect of glycogen on the oxygen and acetoacetate quotients of liver slices from unfasted and fasted rats with and without butyrate is given in Table II.

In liver slices from rats fasted 48 hours the effect of glycogen in the presence and absence of butyrate was investigated. Determinations of acetoacetic acid, β-hydroxybutyric acid, and residual butyric acid were made on the tissue media after the usual 2 hour respiration period. A typical experiment with explanation of calculations is given in Table III.

The results of ten similar experiments (including that recorded in Table III) are summarized in Table IV.

<div align="center">DISCUSSION</div>

The present experiments offer cogent proof that the presence of carbohydrate accelerates the rate at which butyric acid disappears from a medium in which liver slices are suspended. Thus it was shown that the total butyrate as acetoacetate, hydroxybutyrate, and unchanged butyrate remaining in the medium at the end of the tests was less if glycogen had been added to the butyrate substrate.

In a series of ten tests in which slices from the same liver were placed in respirometers containing phosphate buffer solution alone or with added glycogen, with butyrate, or with butyrate and glycogen, it was found that the average butyrate which disappears beyond the ketone body stage after the addition of this component alone was 496 γ per 10 mg. of dry liver, while the level found when glycogen as well as butyrate was added amounted to approximately 594 γ per 10 mg., which corresponds with an increased rate of disappearance of butyric acid of 19.8 per cent over the basal level.

Although there is a considerable variability in the basal rate of disappearance of butyrate in the livers from individual animals, the comparative tests of the speed of disappearance with and without glycogen were carried out simultaneously in each case on slices from the same livers. Despite the considerable differences obtained, the results are significant. This is shown by the fact that in nine out of ten cases the basal rate of butyrate disappearance does not equal the level found when glycogen and butyrate were both present in the medium.

In addition to the greater rate of butyrate disappearance exhibited by the sections treated with glycogen, there is also a marked decrease in the level of total ketone bodies which remain at the end of the test. Although the decrease is not always noted in both of the separate fractions (aceto-

acetate and hydroxybutyrate), it was found that the total ketones in all cases except one were lower than those found in the vessel with butyrate but without glycogen. The average of total ketone bodies in the liver slices immersed in butyrate was 338 γ per 10 mg. of dry liver tissue, while the mean for those tests with slices of corresponding livers to which glycogen had been added had been decreased to 278 γ.

The decreased amount of the ketone bodies with the addition of glycogen must be due to a more rapid disappearance of these constituents. If it were due to a suppression in the formation from exogenous butyric acid, then the butyric acid left at the end of the tests in those cases should be increased. Actually, the total butyrate which still remained unchanged at the conclusion of the experiments was usually slightly less in the glycogen-butyrate medium than in the tests where butyrate alone was used.

Another possible reason which may be advanced to explain the lowered amount of ketone bodies in the carbohydrate-supplemented media is that a decrease in the endogenous ketone formation takes place. Since fewer ketone bodies would then result spontaneously, a lowered content of the acetone bodies might be found, even though the rate of production from butyric acid were unaltered. However, a correction has been made for the endogenous content by subtracting from the results of the butyrate and butyrate-glycogen tests the values of ketone bodies found respectively with liver slices in the basal medium alone and with glycogen.

It has been suggested that the liver slices immersed in the glycogen-butyrate medium might retain enough glycogen to increase the weight of solids. Since the metabolism of butyrate and ketone bodies is based on the weight of the dried liver slices, any such variations might then account for the differences we have noted. To test this hypothesis, the per cent of solids was determined on liver slices after metabolism in media containing butyrate or butyrate plus glycogen. The average total solids found for seven experiments each with both media were identical; namely, 16.0 per cent. The average dry weight of the liver slices was 13.4 and 13.3 mg. for the samples from the butyrate and from the butyrate-glycogen media respectively. That the value for total solids is slightly lower than the accepted value (20 per cent) may be explained by the inability to remove all the adhering physiological saline from the tissue slices prior to the original weighing without danger of damage.

The experiments reported here indicate that the increased rate of disappearance of the ketone bodies in the presence of glycogen cannot be explained on the basis of antiketogenesis. On the other hand, if one understands by "ketolysis" a catalytic removal of ketone bodies by carbohydrate, either by oxidation or by formation of compounds which do not react with Denigès' reagent, these results would seem to support such a mechanism.

It is obvious in the intact animal that the oxidation of any such intermediate compounds may be completed in other tissues than the liver.

SUMMARY

1. The effects of fasting on the acetoacetate content of liver tissue from male rats have been investigated by use of the Warburg technique. The highest and least variable values were reached after a fasting period of 48 hours.

2. For sectioning liver tissue an alternate procedure has been devised which involves the use of a handle constructed of spring metal which holds the ordinary double edged safety razor blade.

3. Quantitative studies made on the effect of glycogen on the oxidation of butyrate by liver slices showed that less butyrate was recoverable as acetoacetic acid, β-hydroxybutyric acid, and residual butyric acid when glycogen was present in the substrate. An increased rate of removal of butyric acid of approximately 20 per cent over the basal level was found in the presence of glycogen.

4. A method for the determination of residual butyric acid in tissue media has been described, a recovery value of better than 93 per cent having been shown.

5. Experiments have been reported which show that glycogen added to tissue media lowers both the endogenous and the exogenous ketone body content of rat liver sections. These results can best be explained on the basis of a ketolytic action.

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DETERMINATION OF THE KETO DERIVATIVES OF CHOLIC AND DESOXYCHOLIC ACIDS IN BILE

By HETTIE B. HUGHES

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A gravimetric method for determining ketosteroids (1) was developed recently in this laboratory. According to this method, trimethylacethydrazide ammonium chloride (Girard's Reagent T (2)) reacts with the carbonyl group (or groups) of the ketosteroid to form a water-soluble steroid hydrazone. This hydrazone is precipitated as the mercuric iodide salt, filtered off, and dried to constant weight.

As shown previously (1), this method gives excellent results when applied to the analysis of highly purified preparations of 3,12-diketocholanic and 3,7,12-triketocholanic acids. It seemed of interest to determine whether the above procedure could be applied to the determination of ketocholanic acids in bile, for according to the observations of Wieland and Kishi (3), Fernholz (4), and Berman and coworkers (5, 6) these keto acids are present in the bile of man, hog, and dog. As the present study shows, the method mentioned above can be applied essentially unchanged to the determination of ketocholanic acids in bile.

EXPERIMENTAL

Experiments were carried out to determine whether 3-hydroxy-12-ketocholanic, 3,12-diketocholanic, 3-hydroxy-7,12-diketocholanic, and 3,7,12-triketocholanic acids[1] could be recovered quantitatively when added to ox bile. There were two reasons for using ox bile in this study: first, according to our own observations, as well as those of Berman and coworkers (5, 6), freshly collected ox bile contains only traces, if any, of the ketocholanic acids; secondly, ox bile contains both conjugated cholic and desoxycholic acids, and it seemed important to know whether the presence of these conjugated acids interfered with the determination of ketocholanic acids.

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[1] We are indebted to Dr. C. W. Sondern and Dr. W. M. Hoehn of George A. Breon and Company, Kansas City, Missouri, for the 3,12-diketocholanic, the 3-hydroxy-12-ketocholanic, and the 3-hydroxy-7,12-diketocholanic acids used in this study.

In brief, the procedure followed in these experiments was as follows: Samples of freshly collected ox bile, to which varying quantities of the above keto acids had been added, were extracted quantitatively with boiling 95 per cent ethyl alcohol, 1 volume of bile being added to 19 volumes of alcohol. An aliquot of such an extract, equivalent to 5 cc. of the original bile, and containing 9 to 30 mg. of ketocholanic acid, was evapo-

TABLE I

Recovery of Keto Derivatives of Desoxycholic and Cholic Acids from Ox Bile

The theoretical weights of mercuric iodide hydrazone were calculated by multiplying the weight of ketocholanic acid taken for analysis by the following factors: 3,12-diketocholanic acid, 4.110; 3-hydroxy-12-ketocholanic acid, 2.547; 3,7,12-triketocholanic acid, 5.503; 3-hydroxy-7,12-diketocholanic acid, 3.987. These factors were calculated according to equations set forth in our earlier report (1).

Ketocholanic acid analyzed	Analysis of pure ketocholanic acid				Analysis of ketocholanic acid added to bile			
	Weight of ketocholanic acid	Weight of mercuric iodide hydrazone		Per cent of theoretical recovered	Weight of ketocholanic acid	Weight of mercuric iodide hydrazone		Per cent of theoretical recovered
		Found	Theoretical			Found	Theoretical	
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	
3,12 - Diketocholanic acid, m.p. 188°	10.1	41.4	41.5	99.8	14.5	60.3	59.6	101.2
	10.1	41.6	41.5	100.1	14.5	59.8	59.6	100.3
	16.0	65.7	65 8	99 8	21.5	88.5	88.4	100.1
	16.0	65.2	65.8	99.1	21 5	87.7	88.4	99.2
3-Hydroxy - 12 - keto-cholanic acid, m.p. 156–157°					25.6	64.1	65.2	98.3
	25.6	64.1	65.2	98.3	25.6	64.8	65.2	99.4
	25.6	64.8	65.2	99.4	30 0	75.0	76.4	98.2
					30.0	76.7	76.4	100.4
3,7,12 - Triketocholanic acid, m.p. 237°	11.9	64.1	65.5	97.8	9.4	51.4	51.7	99.4
	11.9	63.5	65.5	96.9	9.4	50.2	51.7	97.1
	15.1	82.0	83.1	98.7	12.0	65.5	66.0	99.2
	15.1	82.5	83.1	99.3	12 0	66.0	66.0	100.0
					15.2	82.5	83.6	98.8
					15.2	81.5	83.6	97.5
3-Hydroxy-7,12-diketocholanic acid, m.p. 184–185°	16.0	63.4	63.8	99.4	13.0	51.0	51.8	98.4
	17.1	67.8	68.2	99.4	13.0	51.2	51.8	98.8

rated to dryness; the residue therefrom was dissolved in acetic acid-alcohol solution and treated with Girard's Reagent T and mercuric iodide solution as in the procedure for determining ketosteroids described previously (1). The mercuric iodide salt of the bile acid hydrazone thus precipitated was allowed to stand overnight in the refrigerator so as to facilitate filtration, then was filtered off on a Selas filter crucible (porosity 0.01), and dried to constant weight at 104°.

The results obtained in the analysis of bile containing these ketocholanic acids are shown in Table I, together with results on the analysis of similar amounts of the purified ketocholanic acids. Comparison of these data shows that the method gives as good results in the analysis of these acids in bile as in their analysis as pure compounds. Recovery of the pure compounds varied between 96.9 and 100.1 per cent of the theoretical values, whereas recovery of these keto acids from bile varied from 97.1 to 101.2 per cent. It is especially noteworthy that the method is applicable to the determination of both ketohydroxy acids (3-hydroxy-12-ketocholanic and 3-hydroxy-7,12-ketocholanic acids) and keto acids (3,12-diketocholanic and 3,7,12-triketocholanic acids).

Additional experiments were carried out in which the various ketocholanic acids were added to volumes of ox bile ranging from 0.6 to 5 cc. These experiments showed that recovery of the keto acids was essentially the same from any volume of bile within the above range. Recovery from larger volumes than 5 cc. has not been attempted.

These data show that the method described previously for the determination of ketosteroids is applicable to the determination of keto derivatives of the bile acids in bile. It should be pointed out, however, that in analyzing samples of bile containing keto acids of unknown composition results must be expressed arbitrarily in equivalents of one of the known ketocholanic acids.

SUMMARY

A method described previously for determining highly purified ketosteroids has been applied to the analysis of 3-hydroxy-12-ketocholanic, 3,12-diketocholanic, 3-hydroxy-7,12-diketocholanic, and 3,7,12-triketocholanic acids added to bile. The recovery of these compounds from bile ranged from 97.1 to 101.2 per cent of the theoretical amounts.

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METABOLISM OF THE STEROID HORMONES

II. THE CONVERSION OF α-ESTRADIOL TO ESTRONE AND β-ESTRADIOL BY THE OVARIECTOMIZED-HYSTERECTOMIZED RABBIT*

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The biological relationship between α-estradiol and estrone has been established by the isolation of the latter compound from the urine of normal and ovariectomized female and normal male guinea pigs (2, 3) and man (4, 5) following the administration of α-estradiol. In view of the conclusion of Pincus (6), that α-estradiol is not converted to estrone by the ovariectomized rabbit, it was considered of interest to investigate the fate of α-estradiol in the rabbit by methods involving the isolation of crystalline urinary metabolites. The results of such an investigation are the subject of the present communication and demonstrate that, as in the guinea pig and man, α-estradiol is converted to estrone. Furthermore, this transformation does occur in the absence of both the ovaries and uterus.

We have also been able to isolate β-estradiol from the urine of ovariectomized-hysterectomized rabbits to which α-estradiol was administered. β-Estradiol has thus far been found in nature only in the urine of pregnant mares (7–9). Whether it is synthesized *de novo* in the ovaries, the placenta, or the adrenal cortex of the mare or arises as a metabolite of estrone has not been established. From the urine of normal rabbits to which estrone was administered, Stroud (10) obtained an impure phenolic compound which he believed to be β-estradiol. The work reported here demonstrates that α-estradiol is metabolized to β-estradiol in the rabbit, estrone presumably being an intermediate. The ovaries and uterus are not essential

to the conversion but the possibility that these organs are involved in this transformation in the normal rabbit is not excluded.

Since the completion of this work, Heard, Bauld, and Hoffman (11) have reported, in a preliminary communication, the isolation of estrone and β-estradiol from the urine of intact estrous and ovariectomized-hysterectomized rabbits to which α-estradiol or estrone was administered alone or simultaneously with progesterone.

<div align="center">EXPERIMENTAL</div>

100 mg. of α-estradiol dipropionate (equivalent to 70.8 mg. of α-estradiol) were administered by subcutaneous injection daily for 5 days to each of two adult ovariectomized-hysterectomized rabbits.[1] During the period of injection and for the following 3 days, the urine was quantitatively collected under toluene. After acidification by the addition of 10 cc. of concentrated hydrochloric acid per 100 cc. of urine, hydrolysis and extraction were carried out simultaneously by a modification of the method of Dingemanse, Borchardt, and Laqueur (12). The acidified urine, in 1 liter quantities, was refluxed for 6 to 8 hours with 250 cc. of benzene. This process was repeated once more, with a fresh portion of benzene. The benzene solutions were combined and the solvent distilled under reduced pressure.

The total benzene-soluble portion of the urine was dissolved in ether and treated according to the outline given in the accompanying scheme.

Isolation of Estrone—Fraction I consisted of an orange-colored crystalline residue and contained 225,000 I.U. of estrogenic activity.[2] The material was dissolved in 25 cc. of acetone and adsorbed on a column of activated alumina[3] (10 × 140 mm.). Elution was effected by passing through the column 50 cc. quantities of acetone containing progressively greater concentrations of absolute ethanol. Five fractions obtained by eluting the column with acetone containing 2.5 per cent by volume of absolute ethanol yielded an oily residue which crystallized upon standing. Elution with greater concentrations of ethanol in acetone yielded insignificant amounts of material. Upon crystallization from dilute methanol, a crop of 19.5 mg. of crystals, m.p. 245–254°, was obtained from the combined crystalline eluates. The melting point was raised to 251–256° by one recrystallization from dilute methanol. After drying 16 hours at 100° *in vacuo* over phosphorus pentoxide, the compound melted at 255–257°. When mixed with

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[1] We wish to express our thanks to Dr. H. R. Catchpole for performing the operations.

[2] The method of bioassay is described in Paper I of this series (3).

[3] The activated alumina used throughout this work was procured from the Aluminum Ore Company, East St. Louis, Illinois; it is designated grade A, mesh 40.

a sample of estrone (m.p. 256–258°), the melting point of the mixture was 256–258°. The benzoate melted at 215–217° and did not depress the melting point of a sample of authentic estrone benzoate. All melting points are uncorrected.

Isolation of β-Estradiol—Fraction II contained 56,000 I.U. of estrogenic activity. The orange-colored oil was dissolved in 8 cc. of 80 per cent

Benzene-soluble residue dissolved in 500 cc. ether

Extracted 5 times with 100 cc. quantities of 10% NaOH

Total phenolic and acidic compounds in 10% NaOH

Neutral fraction

Acidified with 16% sulfuric acid and extracted 5 times with 100 cc. quantities of ether

Ether-extracted 5 times with 100 cc. quantities of saturated aqueous $NaHCO_3$

Acidic fraction

Ether-extracted 5 times with 100 cc. quantities of 0.1 N NaOH

0.1 N NaOH-soluble compounds

Ether-washed with water and evaporated

Residue treated with Girard-Sandulesco ketone reagent, trimethylacethydrazide ammonium chloride

Ketonic phenols not extracted from ether solution by 0.1 N NaOH (Fraction I)

Non-ketonic phenols not extracted from ether solution by 0.1 N NaOH (Fraction II)

ethanol. 100 mg. of digitonin in 5 cc. of 80 per cent ethanol were added and the mixture was allowed to stand at room temperature overnight. The digitonide, amounting to about 1 mg., was separated by centrifugation and discarded. The supernatant fluid was evaporated to dryness, dissolved in pyridine, and warmed on the steam bath for 1 hour. Ether was then added slowly and the precipitated digitonin separated by centrifuga-

tion.   The supernatant ether solution was washed with dilute hydrochloric acid, saturated sodium bicarbonate solution, and water.   The ether was evaporated and the residue dried.   The residue was dissolved in 25 cc. of acetone and adsorbed on a column of activated alumina (10 × 140 mm.). Elution of a fraction containing solid material was effected by passing through the column a 5 per cent solution of absolute ethanol in acetone. After two recrystallizations from dilute methanol, a product melting at 209–216° was obtained.   A precipitate of fine needles, m.p. 209–217°, appeared in the mother liquors and was separated.   The two fractions were combined.   After the material was precipitated from solution in ethyl ether by the addition of petroleum ether, the melting point was raised to 215–218°.   The compound was dried *in vacuo* over phosphorus pentoxide at 100° for 16 hours.   It weighed 4.8 mg. and melted sharply at 218–219°.   When mixed with a sample of authentic β-estradiol (m.p. 215–216°) the melting point was 215–216°.   The diacetate melted at 140–141° and did not depress the melting point of a sample of β-estradiol diacetate.

DISCUSSION

Estrone and estriol are generally assumed to be the principal active urinary metabolites of α-estradiol.   The experiments of Heard, Bauld, and Hoffman (11) and those reported from this laboratory ((3), and the present work) support this assumption with respect to estrone.   There is no direct evidence, on the other hand, that estriol is a metabolite of α-estradiol or estrone in the animal body.   To the present time estriol has been isolated only from the human placenta (13) and the urine of pregnant women (14–16).   There is yet no chemical evidence that this estrogen arises by any process other than the direct secretion of the placenta.   We have been unable to isolate estriol from the urine of normal or ovariectomized female and normal male guinea pigs (3) or ovariectomized-hysterectomized rabbits (present work) following α-estradiol administration.   Likewise, Heard, Bauld, and Hoffman (11) could not isolate estriol from the urine of estrous or ovariectomized-hysterectomized rabbits which received α-estradiol or estrone alone or simultaneously with progesterone.

Since in our experiment the β-estradiol isolated represents 19.8 per cent of the total amount of crystalline estrogens recovered, this diol must be considered an important metabolite of α-estradiol in the rabbit.   According to Heard, Bauld, and Hoffman (11) β-estradiol represents, in fact, a major active urinary product of α-estradiol and estrone metabolism in the rabbit, for they were able to isolate 4 to 5 times as much β-estradiol as estrone following α-estradiol or estrone administration.

The low titers[4] obtained by the biological assay of Fraction II (non-ketonic phenols not extracted from ether solution by 0.1 N NaOH) and the formation of a small amount of digitonide in this fraction indicate the completeness with which α-estradiol is metabolized by the rabbit. That the human is not capable of metabolizing α-estradiol so thoroughly is indicated by the work of Huffman, MacCorquodale, Thayer, Doisy, Smith, and Smith (17), who isolated appreciable quantities of α-estradiol from the urine of pregnant women. Furthermore, Heard and Hoffman (5) recovered 3.9 per cent of injected α-estradiol unchanged in the urine of a normal man.

The sample of estradiol dipropionate used in our work was prepared from α-estradiol, synthesized by the catalytic reduction of estrone. It is known that small amounts of the β-isomer are produced during the latter process, and for this reason the question arises as to whether the β-estradiol we have isolated from rabbit urine was administered to the animals as a contaminant of the α-estradiol. Dr. Scholz and Dr. Fischer, chemists of Ciba Pharmaceutical Products, Inc., have assured us, however, that with the three purification and recrystallization processes included in the manufacturing procedure of estradiol dipropionate, their estradiol dipropionate can contain only the smallest trace of β-estradiol dipropionate, if it is present at all.

SUMMARY

In the rabbit, as in the guinea pig and man, estrone is a urinary metabolite of α-estradiol. β-Estradiol also arises from α-estradiol when the latter compound is administered to the rabbit, estrone presumably being an intermediate. These conversions occur in the absence of both the ovaries and uterus, but the possibility that these organs participate in the transformations in the normal animal is not excluded.

We wish to acknowledge our indebtedness to Dr. Oskar Wintersteiner, for the samples of β-estradiol and β-estradiol diacetate; to Ciba Pharmaceutical Products, Inc., for the supply of estradiol dipropionate and estrone benzoate; and to the Schering Corporation for estrone.

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[4] According to our method of assay, α-estradiol is about 4 times more potent than estrone in the ovariectomized mouse. On this basis, Fraction II contained less than 1.4 mg. of α-estradiol if all the activity was due to this compound. However, since 4.8 mg. of β-estradiol were isolated, the maximal possible quantity of α-estradiol present in Fraction II is further reduced.

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THE DETERMINATION OF FIBRINOGEN WITH PROTAMINE*

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This presentation deals with a method for the determination of the fibrinogen of plasma irrespective of the presence of anticoagulants like heparin. As is well known, this can be effected by salting-out methods. The older ones no longer are considered adequate for quantitative determination when fibrinogen is in small amounts or when the available plasma is limited. A more recently developed technique (1) in which sodium sulfite is employed yields results in accord with the Cullen-Van Slyke method for the quantitative determination of fibrin. Reference will be made to this later, as it prescribes a 1:25 dilution of the plasma with resultant fibrin loss.

Qualitative Determination of Fibrinogen

Addition of protamine to blood plasma causes a precipitate. When this is centrifuged and washed, it can be redissolved in 3 per cent saline. The resulting solution coagulates between 54–56° and becomes gelatinous in consistency several hours after the addition of fresh serum (thrombin). These characteristics identify this substance as fibrinogen in so far as this is possible.

Further corroboration of the nature of the protamine precipitate of plasma is available from the fact that no precipitate follows addition of protamine to dilute serum. Under this circumstance only a slight haze occasionally follows and this is not influenced by centrifugation. The native globulins of serum obviously are not precipitated by protamine. In this respect they contrast with globulins previously salted-out and redissolved. The latter, less stable, are readily precipitated with protamine. This difference in the behavior of native and redissolved globulins supports the view that the process of isolation alters characteristics of these proteins. For the purposes of the method to be presented it is sufficient that native serum proteins are not precipitated by protamine.

Fibrin Determination

For control of the method to be presented plasma fibrin was determined by a procedure described by a number of investigators (Foster and Whipple (4), Cullen and Van Slyke (3), Wu and Ling (12), Jones and Smith (8), Peters and Van Slyke (9)). A dilution of 1 cc. of oxalated plasma[1] in 25 cc. of saline was used. This was recalcified and after 3 hours at room temperature the plasma-saline-calcium mixture was centrifuged for 20 minutes at 2500 R.P.M. A firm fibrin clot was deposited on the bottom of the tube. The supernatant was drained off, the clot and the wall of the tube washed with 30 cc. of distilled water, and the tube again centrifuged. After a second washing the clot was transferred to a Kjeldahl flask with the aid of 20 to 30 cc. of distilled water, digested with sulfuric acid and copper sulfate, and the nitrogen determined in the usual way. It will be noted that this is a slight modification of the Cullen-Van Slyke and the Wu and Ling methods. Duplicate determinations differed only between 1 and 1.5 per cent.

Quantitative Determination of Fibrinogen

For the quantitative determination of fibrinogen 1 cc.[2] of a 1 per cent solution of protamine in saline was added to a mixture of 1 cc. of oxalated plasma (100 mg. of dry sodium oxalate to 20 cc. of blood) in 25 cc. of saline contained in a carefully cleansed 50 cc. centrifuge tube. Within a minute 3 cc. of a 1.58 per cent calcium acetate solution were added and the tube was then placed in the ice chest for 1 hour. Then it was centrifuged for 20 minutes at 2500 R.P.M., the supernatant poured off, 30 cc. of cold distilled water added, the contents again centrifuged for 10 minutes, and this wash water also discarded.

Early in the development of the method it was found that protamine remains in solution on addition of trichloroacetic acid in absolute alcohol and that after exposure to this reagent the fibrinogen disk, previously impregnated with calcium, is rendered firmer. The next step in the method, therefore, was to add 30 cc. of a 10 per cent solution of trichloroacetic acid in absolute alcohol to the centrifuge tube. Then the fibrinogen mat was loosened from the base of the tube with the sharp end of a glass rod. Small particles clinging to the wall of the tube were freed by using the mat as a wiper and after the tube was recentrifuged the alcoholic solution was poured off. The mat was transferred with the aid of 20 cc. of distilled water to a Kjeldahl flask and the last traces of fibrinogen were removed from the tube with 10 cc. of distilled water to which 3 cc. of

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[1] 50 mg. of dry sodium oxalate to each 10 cc. of blood.

[2] For very large amounts of fibrinogen (0.7 gm. and more in 100 cc. of plasma) 2 cc. of the protamine solution are recommended.

concentrated sulfuric acid were added.   The slight heat generated by the addition of the acid to the water facilitated the process.   This acid solution was added to the contents of the Kjeldahl flask, as was a final rinsing of the tube with a small quantity of distilled water.   After addition of copper sulfate digestion and nitrogen determination were made as usual.   The accuracy of the method was considered satisfactory, as parallel tests with samples of the same plasma differed only within ±2 per cent, rarely ±4 per cent.   This is illustrated in Table 1.

TABLE I

Fibrinogen Determination with Protamine

Bloo l sample No.	0 02 N H_2SO_4 (neutralized with NH_4OH)	Average	Deviation
	cc.		*per cent*
1	2.21		
	2.17	2.19	1
2	1.98		
	2.03	2.01	1
3	2.50		
	2.50	2.50	0
4	4.47		
	4.55	4.51	0.9
5	4.71		
	4.74	4.72	0.3
6	2 33		
	2.33	2.33	0
7	2.99		
	3.07	3.03	1.4
8	2.92		
	2.90	2.91	0 35
9	2.98		
	3.09	3.03	1.7
10	2.52		
	2.48	2.50	0.8

In the course of these experiments it was observed that addition of protamine to plasma leads to rapid and extensive fibrinogenolysis.   The 4 or 5 per cent loss in fibrinogen of oxalated normal plasma incubated for 3 hours is increased to 35 per cent in the same period after protamine has been added.   It should be recalled that under these same circumstances of time and temperature lysis of fibrin is not even initiated.   This is illustrated in Fig. 1.

Difficulties in correlation persisted until it was recognized that the temperature after addition of protamine influenced lysis of fibrinogen and did not affect fibrinolysis.   These facts became evident during a particularly

hot season when it seemed advisable to eliminate the influence of temperature by refrigeration of the plasma-protamine mixture at 3° during the 3 hour period believed to be desirable before centrifugation. It then became apparent that fibrinogenolysis after addition of protamine varied directly with the temperature in accordance with enzymatic action.

When fibrinogenolysis was prevented, the fibrinogen content was found to exceed by approximately 20 per cent the fibrin values obtained by the method detailed earlier in this communication. The discrepancy was less marked only when the fibrinogen content of the plasma was particularly
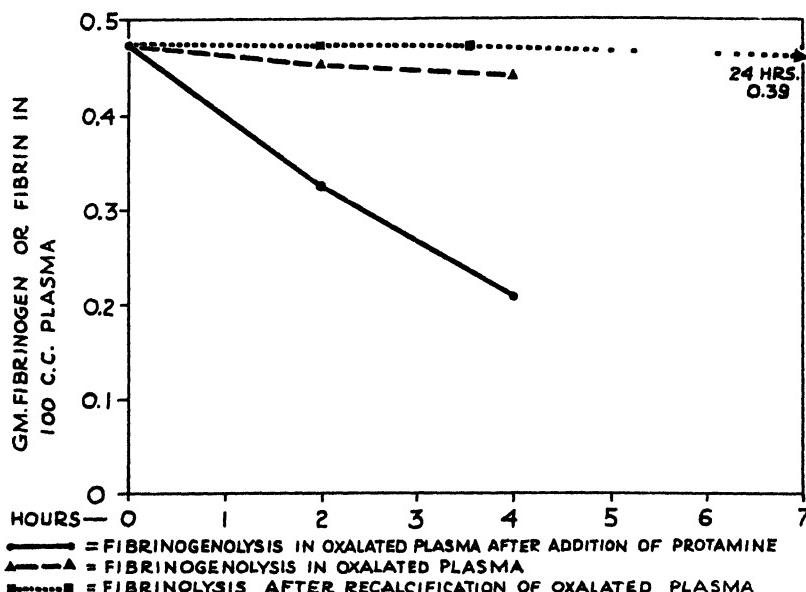
Fig. 1. Marked increase of fibrinogenolysis in oxalated plasma after addition of protamine; fibrinogenolysis in the same plasma without protamine, and fibrinolysis in the same recalcified plasma given as controls.

high. Table II illustrates some of these facts. That the protamine method for the determination of fibrinogen does not yield higher values due to protamine contained in the precipitate is shown by the following experiment. 1 cc. of a fibrinogen solution prepared by repeated precipitation with sodium chloride yielded 4.025 mg. with the Kjeldahl and 3.920 mg. with the protamine method.

Similar discrepancies between fibrinogen and fibrin have been recorded by Jacques (5). Utilizing a fibrinogen solution and purified thrombin, he showed that the total fibrinogen nitrogen could not be recovered as fibrin nitrogen. He concluded that "owing to the solubility of fibrin,

however, as much as 10 per cent of the fibrin nitrogen may be present in the supernatant."

Experiments to elucidate this problem were carried out and typical examples are recorded in Table III. These two experiments show that the samples with the least dilution yielded the highest fibrin values. It is improbable that this is dependent upon impurities included in the clot. Theorell and Widström (10) used 1 part of plasma to 3 parts of diluent as

TABLE II

Discrepancies between Fibrin and Fibrinogen Values

Sample No.	Fibrin; 1 cc plasma, 25 cc saline, 3 cc calcium acetate 0.1 N	Fibrinogen; protamine method, 3°	Fibrinogen-fibrin discrepancy
	gm. per 100 cc plasma	*gm per 100 cc. plasma*	*per cent*
1	0.315	0.383	18
2	0.273	0.352	22
3	0.350	0.437	20
4	0.717	0.804	11
5	0.417	0.530	21
6	0.357	0.439	19

TABLE III

Influence of Amount of Diluent on Fibrin Yield

Sample No.	Oxalated plasma	Calcium acetate 1 58 per cent	Saline	Total fluid	Titration values, 0 02 N NH₂SO₄	Fibrin content
	cc.	*cc.*	*cc*	*cc.*	*cc*	*per cent*
1	1	3	8	12	1.95	0.341
2	1	3	16	20	1.66	0.280
3	1	3	25	29	1.63	0.277
4	1	3	40	44	1.58	0.277
5	1	3	8	12	1.48	0.259
6	1	3	16	20	1.21	0.212
7	1	3	25	29	1 16	0.203
8	1	3	40	44	0 98	0.172

compared to a minimum of 1 to 12 in the above experiments, and their results even with such concentrations were remarkably uniform.

The higher values, it would seem, more nearly approximate the actual content of fibrin and dilution results in substantial loss. This is not predictable, for it is not based upon solubility alone, as is indicated by analysis of the experiments in Table III. The increase in dilution between Samples 1 and 2 is 8 cc. and between Samples 1 and 4 is 32 cc. The corresponding losses in fibrin per cc. of plasma are 0.61 mg. and 0.64 mg.

In the second group the increase in dilution between Samples 5 and 6 is 8 cc. and between Samples 5 and 7 is 17 cc. and the corresponding losses in fibrin per cc. of plasma are 0.47 and 0.56 mg.

More important is the fact that the higher fibrin values obtained with the lesser dilution differ only slightly from those secured with the prota-mine fibrinogen method. The difference is only ±5 per cent. Dilution decreases the fibrin value but the way this is effected is not understood. It does not act by impairing the enzyme system, thrombin, as the following experiment shows.

5 cc. of oxalated plasma were diluted with 125 cc. of saline and recalcified with 15 cc. of 1.58 per cent calcium acetate; 3 hours later the well formed clot was removed and 10 cc. of fresh serum were added. This excess of thrombin did not cause reclotting and it was obvious that the low fibrin value of the dilute solution could not have resulted from lack of thrombin.

The question arises whether dilution may influence the amount of fibrin formation by interfering with the linkage of molecules essential for thread formation. That there are intermediate substances between fibrinogen and fibrin has been shown by Hammarsten (11) and more recently by Apitz (11). Hydration or greater dispersion of intermediate substances or increase of the intermolecular space may impair the final steps. Since fibrinogen precipitation with protamine is largely independent of dilution, it may aid in the solution of this problem.

It is important to note that the difficultly soluble compound of protamine and heparin (Chargaff and Olson (2)) does not interfere with the quantita-tive determination of fibrinogen. When protamine is added to a mixture of plasma and heparin, the fibrinogen is precipitated just as though no heparin were present. The quantity of heparin in the plasma may be in considerable excess of the physiological and indeed may be greater than occurs in anaphylactic shock as determined by Jacques and Waters (6). Moreover, such amounts of heparin influence the nitrogen content of the precipitate so slightly that the fibrinogen nitrogen determination is not interfered with. Parallel experiments without heparin and with 27.5 units for each cc. of blood only show differences of from 1 to 3 per cent.

Precipitation of heparin by protamine has been applied successfully by Jacques and Waters (7) for the quantitative determination of blood heparin. Small amounts of protamine shorten the coagulation time of heparinized blood, while larger amounts, as the authors point out, prolong the clotting time; and as the quantity of protamine is increased the blood may become incoagulable. This latter effect undoubtedly results from the partial precipitation of fibrinogen. The coagulation time of the blood is prolonged with less than 0.5 mg. for each cc. of plasma, owing probably to its influence on fibrinogen. With 0.75 mg., clot formation is entirely inhibited and with

1.5 mg. per cc. of plasma approximately 80 per cent of the total fibrinogen content is precipitated. As a rule 9 mg. of protamine will precipitate all of the fibrinogen but even 18 mg. will not precipitate other nitrogenous substances in the plasma.

On the basis of the above experiments the amount of protamine elected for the quantitative determination of fibrinogen was 10 mg. per cc. of plasma except when the fibrinogen is in great excess, when 20 mg. per cc. are recommended.

SUMMARY

1. A method is described for the quantitative determination of fibrinogen in plasma with the aid of protamine.

2. Heparin does not interfere with this determination when it is present even in the higher amounts shown to occur in anaphylactic shock (25 units per cc. of blood).

3. Fibrinogen values secured by this technique are about 20 per cent higher than fibrin values obtained by the methods of Cullen and Van Slyke and others that require high plasma dilution.

4. These differences are reduced to about 5 per cent if the comparative fibrin determinations are made with smaller plasma dilutions.

5. The discrepancies are not explained by the assumption of a soluble form of fibrin.

6. After the addition of protamine to plasma the enzymatic fibrinogenolysis is markedly increased. About 35 per cent of the fibrinogen is split off after 3 hours incubation of the fibrinogen-protamine precipitate.

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ELECTROPHORETIC STUDY OF THE BLOOD PROTEIN RESPONSE IN TUBERCULOSIS*

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It is well known that there may be definite changes in the relative amounts of the different blood proteins during tuberculosis. An extensive study on this subject was made by Eichelberger and McCluskey (1). They found that changes occurred with progression of tuberculosis in the following sequence: first an increase in fibrinogen, then an increase in globulin, and finally a decrease in albumin. In harmony with many other investigators they found a decrease in the albumin to globulin ratio. This result was confirmed more recently by Molnár (2) and the increase in fibrinogen by Ham and Curtis (3).

All of these results were obtained through the use of precipitation methods and the limitations of such methods for making a true differentiation of the different blood proteins have been demonstrated by the use of the Tiselius electrophoresis apparatus (4-6). Tiselius showed (4), for example, that normal serum consists of several well defined proteins, such as albumin and three different globulins, which he named α, β, and γ, and that the familiar terms "pseudoglobulin" and "euglobulin" represent mixtures of varying proportions of these components. This work opened a whole new approach for more accurate and informative investigation of the serum proteins and has been followed by a great many studies on normal and pathological sera (7-11). Not only has it been possible to determine in this way the relative amounts of the various blood proteins but new components are readily detected. In particular, the antibodies in pneumonia (7, 8) and a few other conditions (12, 13) have so far been identified.

It seemed worth while to undertake an investigation of the blood proteins in tuberculosis by this newer method. The only investigation on tuberculous sera with the newer physicochemical methods which has come to

TABLE I

Variation in Serum Proteins with Development of Tuberculosis

Diagnoses at death: Rabbits R-I and R-II, massive caseous pneumonia; Rabbit A5-19, extensive tuberculosis; tubercles on pleural surfaces, intestines, kidneys, testes, and cerebellum; Rabbit A5-5, small amount of tuberculosis in lungs and larynx; Rabbit A5-20, no tuberculosis except possibly local lesion on rump; died of nephritis; Rabbit A7-8, chronic tuberculosis of lungs, costal pleura, cervical, and axillary lymph nodes, kidney, and intestine; much pulmonary edema; Rabbit A6-6, chronic tuberculosis, mild in lungs and kidneys; died of bronchopneumonia; Rabbit C4S-26, generalized tuberculosis; Rabbit 60, extensive tuberculosis in lungs and pleura; some tuberculosis in intestines, kidneys, and liver; Rabbit 62, extensive tuberculosis in lungs, pleura, kidneys, intestines, and abdominal cavity.

Inoculation with bovine (Ravenel) tubercle bacilli	Rabbit No.	Interval from inoculation to bleeding	Total concentration	Per cent of total as				Presence of X component	Interval from infection to death
				Albumin	α-Globulin	β-Globulin	γ-Globulin		
		days	*per cent*						*days*
Average of 12 normal			5.4	76.0	1.1	10.8	12.2	—	
Ranges			4.5–6.2	72.2–81.5	0–2.5	8.4–12.9	8.4–14.3		
0.001 mg. intratracheally	R-I	51	5.6	70.4		14.8	14.8	—	82
	R-II	51	4.6	65.4		18.0	16.7	—	69
Vaccinated with dead tubercle bacilli; 1 mo. later 0.2 mg. intracutaneously	A5-19	651	5.8	53.1		31.8	15.1		684
	A5-5	655	5 6	70.4	4.5	13.3	12.1	—	
		713	3.9	63.5	4.7	16.2	15.6	—?	
		771	3.3	50.0	11.0	27.2	11.8	+	779
	A5-20	658	6.5	60.7	3.4	15.0	20.8	—	
		744	5.8	59.0	3.2	12.8	24.9	+	
		771	6.7	57.7	5.0	10.8	26.5	+	
		800	5.3	52.4	6.5	17.6	23.5	+	
		815	5.1	55.1	4.4	21.8	19.7	+	
		856	6.6	41.8	20.1	21.4	16.8	—	861
0.0001 mg. intravenously	A7-8	Before	5.0	73.3	4.2	10.6	12.2	—	
		31	6.1	65.7	3.8	14.4	16.1	—	
		59	6.2	60.4	4.6	17.7	17.3	—	
		72	5.8	60.3	3.2	17.2	19.3	—	106
	A6-6	Before	5.0	72.9	5.1	8.8	13.2	+	
		65	5.0	62.5	6.5	12.0	19.0	—	108
	C4S-26	Before	4.4	72.9	4.4	10.3	12.4	+	
		38	5.7	64.8	7.9	16.0	11.4	+	56
0.2 mg. intracutaneously	60	Before	6.2	76.8	1.4	11.9	10.1	—	
		16	6.2	74.9	3.3	11.2	10.7	+	
		68	6.0	68.3	4.8	14.0	12.7	+	
		117	6.4	57.7	7.8	20.3	14.1	+	
		132	6.3	56.1	4.8	23.8	15.3	—	139
0.2 mg. intracutaneously	62	Before	5.8	76.2	1.6	8.9	13.3	—	
		10	5.3	73.9	0.9	14.3	11.2	+?	
		34	4.8	71.8	3.5	12.0	12.7	+	
		48	6.4	70.9	3.4	13.3	12.5	+	
		57	6.2	68.5	3.0	17.1	11.5	+	
		86	7.4	55.6	4.5	24.9	15.1	Trace	94

our attention was an ultracentrifuge study by McFarlane (14) which indicated that some of the globulins were high.
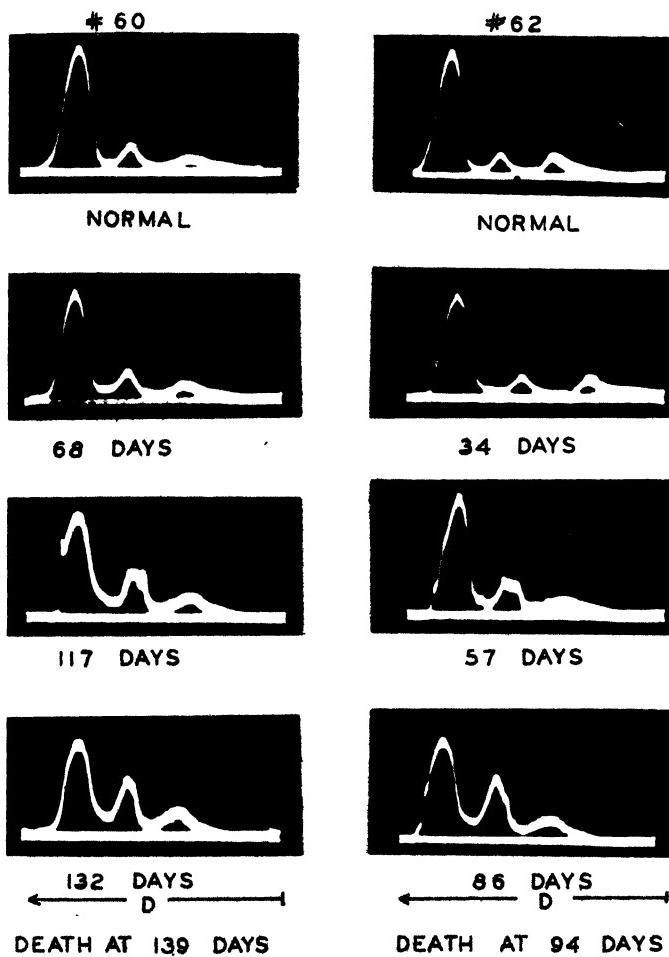
FIG 1. Electrophoretic diagrams of rabbit serum proteins during the development of tuberculosis All exposures are of the descending curves and taken at 30°. Rabbits 60 and 62 were experimentally infected with tubercle bacilli and representative diagrams at various intervals after infection show first the rise in the α-globulin (usually a rise from the base-line), and the X component (a break in the leading side of the albumin curve), and finally a marked rise in β-globulin

In the studies to be reported here serum was used rather than plasma, in order to avoid the possibility of masking an antibody by the presence of fibrinogen. Studies on plasma are in progress. At first the normal

variation in different rabbits and in the same rabbit from time to time had to be determined under carefully standardized conditions. In all cases the whole serum was dialyzed in a heavy cellophane sac for about 3 days against phosphate buffer at pH 7.7, $\mu = 0.1$, and then diluted 1:4 before study at 0.5–1.0°. A potential gradient of about 6.6 volts per cm. was employed for about 90 minutes in all experiments. The Svensson modification of the Philpot optical arrangement (15) was used. In each case the areas under four enlarged curves of the descending boundaries taken at different angles were measured with the planimeter and from their average the concentration of the components was calculated. Each component was recorded in terms of percentage of the total protein. The total protein concentrations were also recorded as the sum of the individual components calculated from the diagrams.
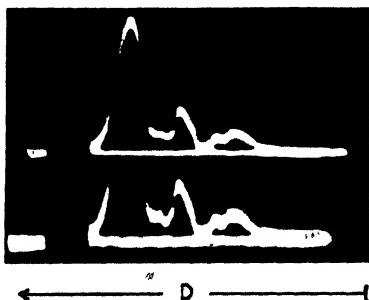
Fig. 2. Descending curves of a tuberculous rabbit serum, showing that at an angle of 40° (top) the α-globulin appears mainly as a rise from the base-line, whereas at a larger angle, 50° (lower), a definite peak can be seen.

It was possible to establish a normal range for the various blood proteins, and thus it is quite simple to detect any deviation from the normal. For example, on several occasions supposedly normal rabbit sera gave values outside of the normal range. Subsequently, at least two of these were found to be due to unsuspected pregnancies, two to sore hocks, and one to nephritis. Table I gives the average percentages found for the different proteins in normal rabbit serum, as well as the ranges of percentages.

The values found at different times for the blood proteins in ten tuberculous rabbits are also given. These rabbits have all been examined at autopsy, and similar results are being obtained on experimentally infected rabbits which are still living. In five of the ten rabbits an analysis was made before inoculation and showed normal values. Fig. 1 gives the electrophoretic diagrams showing the progressive changes in the serum of two representative rabbits during the development of tuberculosis.

In all cases the percentage of albumin was lower than in the normals and

progressively decreased with progression of the disease, while the globulins increased. The first changes which occurred were usually a rise in the

RABBIT SERUM
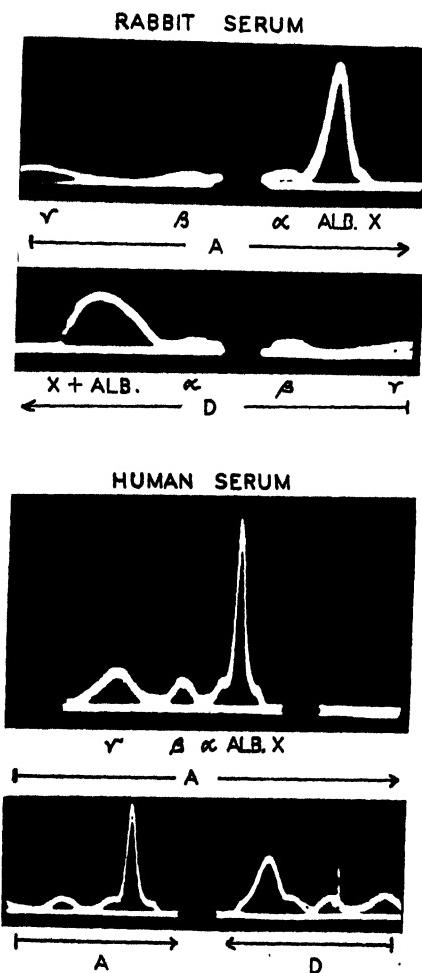
HUMAN SERUM

FIG. 3. The X component and α-globulin fraction show distinctly in both examples of rabbit and human tuberculous serum. *A* represents ascending curves and *D* descending curves and the arrows indicate the direction of migration. The human serum diagrams represent different exposures taken of the same serum (R. B.) as the last diagram on Fig. 4. Both exposures of the rabbit serum were taken at 40° angles, and the first exposure of human serum at 30° and the last at 20°.

α-globulin fraction and the appearance of an unknown component which will be designated the X component, especially if the disease progressed

slowly. This rise in the α-globulin fraction appears in Fig. 1 in many cases simply as a rise from the base-line in the curve between the albumin and β-globulin. Compare the diagram for normal serum. In many cases, if the angle is made large enough, as illustrated in Fig. 2, a definite peak becomes evident.

In some cases the X component appeared as a distinct gradient, as seen in Fig. 3 especially in the ascending diagrams, while in other cases it was seen as a break in the leading side of the albumin curve, as in Fig. 1 (117 days, Rabbit 60, and 34, 57, and 86 days, Rabbit 62). In many cases, this component did not separate distinctly on the descending side, as seen

<div align="center">

TABLE II

Serum Proteins in Normal and Tuberculous Human Beings

</div>

Seia	Total concentration		Per cent of total as				Presence of X component
			Albumin	α-Globulin	β-Globulin	γ-Globulin	
		per cent					
Normal	Average of 5	6 5	66.3	4 5	12 2	17 0	$+$*
	Range	5.0-7 5	63 7-68.9	3.4-5.7	9 8-13.8	14.9-19 3	
Tuberculous	A. H.	7 5	53 7	4 1	13 7	28 6	—
		7 8	53.0	5.8	13 8	26 9	$+$
		7 3	52 8	5.1	15 2	26 9	$+$
	R. B.	6 5	54.8	9.0	13 6	22 5	$+$
		7 0	53.5	7 6	15 2	23.7	$+$
		9 0	52.9	8 1	15 5	23 6	$+$
	L. V.	7.2	58.6	6 5	14.3	20 6	$+$
		6.5	58.9	5.9	15 9	19 3	$+$
		8 1	58 7	7 1	13.5	21.0	$+$

* In one subject.

in the rabbit serum of Fig. 3, but caused the albumin curve to become very heterogeneous in shape.

The mobility of this X component was slightly greater than that of albumin (-7.1 to -7.3×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ on the descending side). It is interesting that this mobility is the same as that found under identical conditions for a protein component in the purified tuberculin protein. The suggestion thus arising, that the tuberculin protein may exist in the serum as such, is further supported by the following experience. When tuberculin protein was added to a serum which originally showed no evidence of the component, it did then appear in the same manner as seen in the sera mentioned above. It is probable in this case that this component represented the excess tuberculin protein. Furthermore, the X

component appeared regularly in normal rabbits after sensitization with tuberculin protein (16).

The percentage of α-globulin always increased in normal rabbits after sensitization to the tuberculin protein and evidence was presented (16) to show that it was at least partly due to the tuberculin protein antibody.
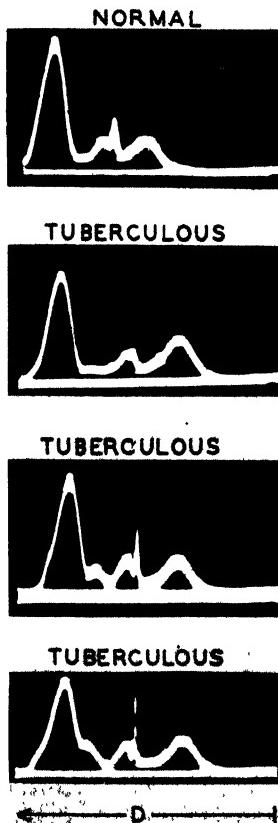
FIG. 4. Human sera, descending curves. The large γ-globulin is seen in the first diagram of tuberculous serum (A. H.). The last two sera (L. V. and R. B.) show the prominent α-globulins and X components. All exposures were of descending curves taken at a 30° angle

The presence of this antibody, however, was not associated with immunity (17). The increase in α-globulin in early tuberculosis may therefore be evidence of sensitization to the tuberculin protein.

One of the most constant results found during the development of tuberculosis was an increase in the β-globulin before death; in some cases

this increase was very great (see Table I and Fig. 1). In many, but not all cases, a split occurred in the β component, but it is well recognized (5, 9, 10) that this component is not homogeneous.

Table II shows that in a preliminary series similar blood serum changes occur in human tuberculosis. Three patients with far advanced tuberculosis were studied. The albumin in all cases was low and the α- and γ-globulins high (see Fig. 4). The X component also appeared, whereas it was not present in four of five normal human sera examined. The one normal person whose serum did show it was known to have been sensitized to the tuberculin protein from constant contact with it.

There is a suggestion in this work that a high γ-globulin may be associated with resistance to the disease. For example, Rabbit A5-20 was reported by Dr. Lurie to have died, not from tuberculosis, but from nephritis. The local lesion at the site of inoculation had completely healed, and the only other lesion of a tuberculous character was a large draining pus sac on the rump. It was not possible, however, to identify tubercle bacilli in this pus either by staining or by inoculation into a guinea pig. The γ-globulin was exceptionally high and began to decrease before death, at which time the β-globulin increased greatly.

Furthermore, in the case of patient A. H. the γ-globulin was very high when the first test was made, as seen in the first diagram of tuberculous serum of Fig. 4, and at this time he had been resisting an extensive tuberculosis for at least 3 years, during which time he had spent 1 year in a penitentiary. At the time of the second and third tests made 5 months after the first test and within an interval of 3 weeks, he was reported to be doing poorly, and the γ-globulin was decreasing. The α-globulin was increasing, and the X component appeared, as seen in Table II. At this time the electrophoretic diagram was similar to the last two curves of Fig. 4.

DISCUSSION

It is obvious from these studies that definite changes occur in the proportions of the blood proteins during progression of tuberculosis and that these proportions are different in early or mild tuberculosis from those in late disease with much involvement. For example, in early tuberculosis a rise in the α-globulin component and the appearance of an abnormal component (designated as the X component), which is slightly more mobile than albumin, occurred. A similar rise in the α-globulin was reported by Blix (8) to occur in pneumonia, and furthermore, he mentioned that at least two distinct boundaries could be seen in the albumin fraction after long continued electrophoresis. Longsworth et al. (10) also reported a high α-globulin in the sera of febrile patients. It has been clearly shown in another publication (16) that a very marked rise in the α-globulin, as well

as the appearance of the X component, is caused by sensitizing normal rabbits with tuberculin protein and even with egg albumin. Therefore, the phenomenon is probably evidence of protein sensitization and may occur in many conditions where such sensitization is involved. It is notable that one of the first recognizable events in tuberculosis is sensitization to the protein of tuberculin.

The rise in β-globulin in terminal tuberculosis is especially significant in view of the fact that Blix, Tiselius, and Svensson have reported this fraction to be a conspicuous lipid carrier. The significance of the rise in this component at a time when it is known that the infected organism contains much tubercle bacillus phosphatide is under definite consideration.

While the electrophoresis technique has been of definite value in following the changes occurring in the blood serum proteins during the development of experimental tuberculosis, it is obvious that it cannot be of much diagnostic value, since similar changes occur in many other diseases. However, it can be of help in estimating prognosis, although it is a time-consuming and laborious procedure from the standpoint of volume of analyses. Its greatest value lay in its use for the study of the fundamental nature of the disease and in the search for antibodies.

SUMMARY

Criteria are given for normal rabbit and human sera which make it possible to determine by means of the Tiselius electrophoresis technique whether subjects are normal or not.

There is always a progressive decrease in the amount and percentage of albumin with progression of tuberculosis.

The first change in the globulins in early tuberculosis is usually a rise in the percentage of α-globulin. This is generally accompanied by the appearance of an abnormal component, here designated the X component, a fraction with a mobility slightly greater than albumin. These changes are probably evidence of sensitization to the tuberculin protein.

The β-globulin in all cases increased as the disease reached the terminal stage.

Some evidence, although so far insufficient for definite conclusion, indicates that a rise in the γ-globulin may accompany resistance to the disease.

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CHEMICAL COMPONENTS OF SOME AUTOTROPHIC ORGANISMS*

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In an earlier study of the amino acids in the total proteins of autotrophic organisms (1) it was found in a group of marine algae that the most primitive species lacked tyrosine, lysine, and methionine, and that these amino acids appeared, in the above order, on ascending the evolutionary series. On the other hand, a fresh water alga, *Phormidium*, was found to be deficient in cystine, which was present in the marine algae.

This study has been extended to other autotrophic organisms, of which the content of certain of the protein amino acids, the lipids, and other components has been examined. The results set forth in Table I indicate[1] the absence of individual protein amino acids in certain species, but display less regularity than was found with the marine algae previously examined. In the blue-green group, *Gloeotrichia*, like *Phormidium*, is devoid of cystine; *Caulerpa* (green), like *Ulva* (green), *Phormidium* (blue-green), and *Laminaria* (brown), contains no lysine. A deficiency not encountered hitherto is the absence of arginine from the proteins of the brown algae *Fucus*, *Cystoseira*, and *Egregria*. The difference in amino acid composition between *Fucus* and *Sargassum* (in which the absence of only methionine has been demonstrated (1)) is striking in view of the close phylogenetic relationship of these two species. On the other hand, the less closely related pair of algae *Caulerpa* (green) and *Laminaria* (brown) has in common the absence of lysine, though the latter alone lacks methionine.

The lipid composition, determined by the methods outlined in a recent report (2), is recorded in Table II. The presence of free fatty acids may be ascribable to the fact that the specimens were dried in air after collec-

[1] It is conceivable, though improbable, that the apparently complete absence of cystine from the hydrolysate of one alga and of arginine from others may be due to decomposition of these amino acids by the action of unidentified components of the organisms during hydrolysis. Scarcity of material unfortunately prevented suitable control experiments.

tion. Differences in the quantitative distribution of the various lipid fractions were observed with the different species, but no obvious generalization is indicated.

In concordance with the results of previous investigators (3) mannitol was isolated in large amounts (10 per cent) from *Laminaria* and in smaller yields from other brown algae: *Macrocystis* 3.4 per cent, *Lessoniopsis* 2.4

TABLE I

Percentage Distribution of Formic Acid-Soluble Nitrogen

	Gloeo-trichia	Macro-cystis	Lesson-iopsis	Fucus	Cysto-seira	Egregria	Caulerpa	Codium	Di-atoms
Acid humin .	8.2	9.9	9.5	8.6	7.3	5.0	10.2	9.8	5.7
Ammonia.... .	6 4	4.0	2.8	7 2	3.8	3.5	6 4	8.2	1.1
Tyrosine . .	0.9	0 3	0.6	0.8	0.9	0.7	1.3	0.5	0.2
Tryptophane	0.3	0.5	1.9	0.5	0.8	0 9	1.9	0.4	6.2
Cystine.. .	0 0	0.6	1.1	1 6	2.2	0.6	0 6	0.5	0.6
"Methionine"*	14 3	17.0	3.4	5.5	6.2	2.5	1.6	2.3	2.4
Histidine .	1.5	1.5	1.2	1.0	4.3	4.1	3.1	4.5	8.4
Arginine .	2.6	8.8	3.2	0.0	0.0†	0 0†	6.0	7.2	1.7
Lysine	2 0	1.9	7.7	7.2	4.1	0.4	0.0	5 3	4 4

* Organic non-cystine sulfur; obtained by the difference between total sulfur and alkali-labile sulfur, calculated as methionine N.

† Gave a faintly positive Sakaguchi test, but no flavianate.

TABLE II

Percentage Distribution of Lipids in Algae

	Gloeo-trichia	Macro-cystis	Lesson-iopsis	Fucus	Cysto-seira	Egregria
Total lipid* . . .	4 0	1.7	4.6	2 1	1.4	1 1
Saturated acids (free).	20.0	2 1	0.2	4.1	8.2	2.5
Unsaturated acids (free)	26.3	23.7	0.7	29.7	2.1	22.0
Saturated acids (combined)	0.6	1 3	8.3	0.8	0.8	4.1
Unsaturated acids (combined)	4 6	5 7	1.6	7.3	10.2	15 6
"Hydrocarbons"† .	3.4	20.5	56.8	27.9	12.2	27.0
Alcohols .	21.5	6 9	3.4	7.6	13.1	9.2

* Per cent of the air-dried algae soluble in petroleum ether.

† Fraction which formed no ester with succinic anhydride; might contain non-alcoholic ketones.

per cent, *Fucus* 2.2 per cent, *Cystoseira* 1.5 per cent, *Egregria* 0.8 per cent, and none from the green algae *Ulva*, *Caulerpa*, and *Codium*.

EXPERIMENTAL

Specimens of *Egregria menziesii*, *Lessoniopsis littoralis*, *Macrocystis pyrifera*, *Cystoseira osmundaceae*, *Codium fragile*, and *Fucus furcatus* were kindly collected by Dr. G. M. Smith of Stanford University at Pacific

Grove, California, in January, 1938. These samples were dried at 34° immediately after collection; the average weight was 450 gm. *Caulerpa racemosa* (230 gm. dry weight) was collected by Dr. Hugh H. Darby of this laboratory in July, 1938, from the moat walls of Fort Jefferson in the Dry Tortugas; the material was immediately rinsed in fresh water and covered with alcohol. *Gloeotrichia echinulata* (289 gm. dry weight) was collected by Dr. G. L. Clarke of Harvard University in August, 1938, at Arbor Vitae Lake, Wisconsin; it was immediately covered with acetone. We are greatly indebted to these gentlemen for their cooperation.

These materials were subjected to essentially the same series of operations. The first step was a continuous extraction with hot ethyl alcohol. The solid which separated from the cold solution was recrystallized from alcohol; insoluble inorganic matter was discarded, and the recrystallized product, when obtained, was identified as mannitol by combustion analysis, by mixed melting point (166–167°), and by preparation of the hexaacetate, m.p. 119°, $[\alpha]_D = +16°$ (1 per cent in pyridine). The total yield of mannitol was secured by systematic recrystallization.

The combined mother liquors were evaporated and extracted with ether. The plant tissue was exhaustively extracted with ether, and the combined ethereal solutions were evaporated to dryness. The residue was treated with petroleum ether, which took up the total lipid fraction. The ether-soluble, petroleum ether-insoluble fraction was discarded.

The proteins were extracted from the lipid-free tissue by means of 90 per cent formic acid, as in the earlier investigation (1). The subsequent steps were essentially the same, except for a simplification of the routine for the isolation of the basic amino acids. Arginine was precipitated with flavianic acid at pH 3; histidine was precipitated from the filtrate with mercuric chloride (4) and isolated as the hydrochloride; lysine was precipitated with phosphotungstic acid and isolated as the hydrochloride.

The lipids were fractionated by the methods outlined in a recent paper (2). Two of the marine algae discussed in the previous report (1) yielded oils from which crystalline sterols were secured.

Sargassum yielded 2.4 per cent of total lipid. The total saturated acids in this amounted to 19.0 per cent, and the unsaturated acids to 31.3 per cent. The unsaponifiable fraction, dissolved in petroleum ether, was repeatedly shaken with 90 per cent methanol, which extracted a crystalline sterol amounting to 5.1 per cent of the weight of the total lipid. After recrystallization this formed needles, m.p. 124°, and $[\alpha]_D = -34.3°$ in acetone; the acetate melted at 119°. Identity of this sterol with fucosterol, previously isolated by Heilbron, Phipers, and Wright (5) from the closely related alga *Fucus vesiculosus*, was kindly confirmed by Dr. Heilbron, by direct comparison.

Ulva lactuca yielded 2.7 per cent of total lipid, which by similar treat-

ment yielded 15.2 per cent of saturated and 33.6 per cent of unsaturated acids, and 1.2 per cent of a sterol. This crystallized in plates, m.p. 137°, $[\alpha]_D = -38.1°$ in chloroform; the acetate, m.p. 133°, had the composition $C_{31}H_{50}O_2$ (calculated, C 81.87, H 11.09; found, C 81.8, H 10.8) and formed a tetrabromide (Br, calculated 41.2, found 42.3) which melted at 148°.

SUMMARY

In continuation of a study of the chemical constituents of primitive autotrophic organisms, amino acid analyses have been made of hydrolysates of the total proteins extracted from five brown and two green algae. Of the former, all contain lysine but three lack arginine; of the latter, one lacks lysine.

The second blue-green fresh water alga to be examined is, like the first, devoid of cystine.

Further confirmation of the apparent generality that mannitol is present in brown marine algae, but not in green, has been secured.

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THE ESTIMATION OF HYPOXANTHINE

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In a previous paper there was described a procedure for the isolation of the purines in a form suitable for the quantitative determination of the individual substances (1). A later publication reported a method for the separation of adenine and hypoxanthine and the determination of the adenine by precipitation of adenine picrate and titration of the precipitate with standard alkali (2). The method to be described in the present paper was designed primarily for the estimation of hypoxanthine in the filtrate from the adenine picrate, but is applicable without modification to other analytical problems.

The method involves the precipitation of the hypoxanthine silver picrate first described by Bruhns (3) and ultimately the determination of the silver in the precipitate by titration with standard thiocyanate solution. Because hydrochloric acid is superior to other acids in the decomposition of the cuprous purine complex (1), it is necessary to separate the hypoxanthine silver picrate from silver chloride precipitated simultaneously. This is accomplished by the extraction of the hypoxanthine compound with hot concentrated nitric acid in which silver chloride is practically insoluble. This means of separation and the final volumetric estimation are improvements which allow a considerable extension in the use of the argentipicrate procedure. This method heretofore has been excluded in the presence of chloride and has required large samples of material. Under the conditions described herein, 1 mg. of hypoxanthine nitrogen (18 micromoles) can be determined with an error of about 1 per cent, and the error is not greatly increased when only 0.5 mg. of hypoxanthine nitrogen is available for analysis.

Procedure

The solution to be analyzed, containing 1 to 5 mg. of hypoxanthine nitrogen in a volume of about 6 ml. is placed in a large (200 $\times$ 25 mm.) Pyrex test-tube with a pouring spout. If picrate is not already present as in the adenine filtrate (2), add 2 ml. of saturated sodium picrate solution. Add 3 ml. of N nitric acid, place in a boiling water bath, and, when hot, add dropwise 1 ml. of 0.2 N silver nitrate (0.5 to 1 ml. in excess of the amount required

to precipitate the chloride). Continue to heat for 5 minutes; then allow the solution to cool slowly to room temperature (30 minutes). Filter with suction through an asbestos pulp mat in the 2.5 cm. filtration tube described by Fiske (4), or a filter tube with a fritted glass disk, retaining the greater part of the precipitate in the test-tube. Wash with several 5 ml. portions of water to remove picric acid completely. Extract the precipitate with 3 ml. of hot concentrated nitric acid. Pour the solution through the filter and receive in a second large test-tube. Complete the transfer with two additional 2 ml. portions of hot nitric acid. Add 1 ml. of 10 N sulfuric acid and evaporate to about 1 ml. by the use of heat and a stream

TABLE I

Effect of Acidity on Precipitation of Hypoxanthine Silver Picrate

Experiment No.	No of determinations	Acid present	Recovery		Description of precipitate
			Range	Average	
		N	*per cent*	*per cent*	
1	1	0.0006		122.2*	Amorphous
2	3	0.02 –0.09	99 6–100.2	99.9	Needles
3	1	0.033		139.5*†	Amorphous
4	2	0.033	100.2–100.5	100 4	Needles
5	5	0 12 –0.25	99.6–100.2	99 9*	" and platelets
6	4	0.30 –0 47	99.6–101 0	100 5	" " "
7	3	0.58 –0 82	99.6–100.2	100.0‡	Platelets
8	5	1.0	98.7–100 3	99.9‡	"
9	3	2 0	97 5– 99 0	98.3‡	Fine white needles
10	1	4 0		81.7‡	" " "

* Gravimetric determinations on replicate samples gave the following recoveries, calculated as hypoxanthine silver picrate: Experiment 1, 88.8 per cent, Experiment 3, 90.6 and 93.8 per cent, Experiment 5, 100.0 and 101.2 per cent.

† Cooled immediately after addition of silver nitrate.

‡ Allowed to stand 1.5 hours or longer at room temperature.

of hot air (5). Complete the destruction of the organic matter by digestion to fumes over a micro burner flame, with the addition of 1 or 2 drops of nitric acid. Cool, and add 3 ml. of water and 1 ml. of saturated ferric ammonium sulfate. Titrate with standard 0.01 N ammonium thiocyanate solution. From the titer subtract the quantity of thiocyanate required in a blank titration (about 0.02 ml.). 1 ml. of 0.01 N thiocyanate is equivalent to 0.56 mg. of hypoxanthine nitrogen.

Acid Concentration—Hypoxanthine silver picrate exists in two crystalline modifications (Table I). When it is precipitated from solutions in which the acidity is less than about 0.1 N, it crystallizes as the orange-colored needles described by Bruhns (3). When the acidity is greater than about

0.5 N, the precipitate takes the form of thin, shining platelets somewhat more yellow in color. At intermediate acidities a mixture of the two modifications is formed. When the acid concentration is less than about 0.01 N, the precipitate is amorphous. The amorphous substance has a silver-hypoxanthine ratio greater than 1, and a lower molecular weight than hypoxanthine silver picrate. Presumably it consists, in part at least, of hypoxanthine silver in which the ratio is 2. The precipitate also is amorphous if the solution is cooled immediately after the addition of silver nitrate, even though the acidity be great enough for the formation of the crystalline argentipicrate. Hence the solution must be hot when the silver nitrate is added and the subsequent cooling must be slow to allow the crystallization of the argentipicrate.

Separation of Hypoxanthine Silver Picrate and Silver Chloride—Hypoxanthine silver picrate is converted to hypoxanthine silver nitrate by the action of strong nitric acid. The solubility of the latter was determined in nitric acid solutions of various concentrations and was found to increase rapidly with increasing nitric acid concentration. At the temperature of the boiling water bath, the solubility (S) in mM per liter can be expressed by the following equation, log $S = 0.94 + 0.072$ N, where N is the normality of the nitric acid. The agreement between solubilities found and calculated by the equation may be illustrated by the following examples: normality 1, calculated 10.3, found 10.0; normality 15, calculated 105.0, found 102.0. The solubility of silver chloride, on the other hand, was found to decrease with increasing nitric acid concentration. For example, at the temperature of the boiling water bath the solubility was found to be 0.22 and 0.020 mM per liter in 4 N and concentrated nitric acid respectively. The extraction of hypoxanthine silver picrate from silver chloride is most efficient, therefore, when concentrated nitric acid is used.

Other Purines—*Guanine* and *adenine* interfere with the determination of hypoxanthine, as both form insoluble silver compounds, and must be removed before the determination of hypoxanthine is attempted.

Uric acid is destroyed by heating with the concentration of nitric acid employed in the precipitation of hypoxanthine (6) and does not interfere.

Xanthine forms a silver compound (7) which might be expected to precipitate under some circumstances. The maximum concentrations of xanthine which introduce no significant error in the hypoxanthine determination are shown by the data of Table II. When the concentration of nitric acid is 0.25 N, as the method was described, the allowable limit of xanthine is about 0.5 mg. of nitrogen. By raising the acid concentration, the allowable limit of xanthine is increased to 2 (0.5 N) or 3 (N) mg. of nitrogen, sufficient to allow a quantitative separation of the two purines. Hypoxanthine can be recovered quantitatively at the higher acid concen-

trations provided an extended time (1.5 hours) for crystallization is allowed. However, the precipitates are more difficult to manipulate because, under these conditions, considerable amounts of picric acid precipitate. The higher concentrations of acid are used therefore only when a quantitative xanthine determination (6) has shown this modification of the method to be necessary.

TABLE II

Effect of Xanthine in Hypoxanthine Determination

2.00 mg. of hypoxanthine nitrogen were present in each determination. The solutions were allowed to stand 1.5 hours before filtration.

Xanthine added	HNO$_3$ present	Hypoxanthine found	Error
mg. N	*N*	*mg. N*	*per cent*
0.3	0.25	2.00	0.0
0.4	0.25	2.03	+1.5
0.5	0.25	2.08	+4.0
0.6	0.25	2.16	+8 0
1.0	0.50	2.02	+1.0
2.0	0.50	2.00	0.0
3.0	0.50	2.38	+19.0
4.0	0.50	3.18	+59.0
2.0	1.0	2.01	+0.5
3.0	1.0	1.98	−1 0
4.0	1.0	2.05	+2.5
5.0	1.0	2.47	+23.5

Results

Table III gives the results of a number of determinations of hypoxanthine in solutions from which the adenine had been separated by the method described earlier (2). In the first six experiments, a mixture of the purines was evaporated with an excess of hydrochloric acid, and the purine hydrochlorides were used as the starting material. The average recovery in these experiments amounts to 100.4 per cent. The last four experiments illustrate the recoveries which are to be expected when hypoxanthine is determined in tissue extracts and other materials which require a preliminary isolation of the purines by means of a copper precipitation (1). The average recovery in these experiments was 98.3 per cent. This may be compared with an average recovery of 98.7 per cent, based on nitrogen determinations, when hypoxanthine alone was carried through precipitation with copper and bisulfite and decomposition of the precipitate (1). It may be concluded that hypoxanthine can be determined with approximately the same accuracy in an adenine picrate filtrate as when hypoxanthine alone is taken.

In Table IV are given a number of representative experiments which illustrate the application of the method to tissue extracts. The preliminary isolation of the purines and the determination of adenine and xanthine were

TABLE III

Determination of Hypoxanthine in Adenine Filtrate

Purine N present		Hypoxanthine found
Adenine	Hypoxanthine	
mg.	*mg.*	*per cent*
0.994	0.997	100.5
0.994	2 001	101 0
0.994	3.012	100.4
2.002	0.997	99.4
2.002	2.001	100.2
2 002	3.012	100.6
0.997	2.001	97.1*
0.995	3.012	98.4*
2.001	0.995	98.9*
3.012	0.997	98.7*

* After isolation of the purine hydrochlorides by means of a copper-bisulfite precipitation (1).

TABLE IV

Determination of Hypoxanthine in Tissue Extracts

Experiment No.	Tissue	Purine found		
		Adenine	Hypoxanthine	Xanthine (guanine)
		mM per kg. tissue	*mM per kg. tissue*	*mM per kg. tissue*
1A	Rat muscle	6.17	0.0	(0.11)
1B		0.29	5.18	1.02
1C		0.17	5.24	0.98
2A	" Carcinoma 256	1.30	0.0	(0.04)
2B		0.14	0.23	1.87
2C		0.20	0 18	1.12
3A	Mouse Sarcoma 180	2.85	0.0	(0.34)
	Same, 2 hrs. at 38°	0.93	1.20	2.43

In the experiments A represents fresh tissue; B, tissue after 2 hours incubation at 38° with 2 volumes of 0.24 N $NaHCO_3$; C, same as B but containing NaF, 0.1 N.

described in earlier papers (6, 2). In the first experiment (No. 1A) a sample of rat muscle was removed from an anesthetized animal and quickly frozen with CO_2 snow. This sample was found to contain 6.17 mM of adenine, a negligible amount of hypoxanthine, and 0.11 mM of chromogenic

purine (guanine) respectively per kilo of tissue. Aliquot samples of the same muscle then were incubated with 2 volumes of 0.24 N sodium bicarbonate without (Experiment 1B) or with (Experiment 1C) the addition of sodium fluoride at 0.1 N concentration. The adenine was nearly completely deaminized during autolysis. The greater part of the product was found to be hypoxanthine (5.18 and 5.24 mM per kilo), but some xanthine was formed (1.02 and 0.98 mM per kilo). In each instance the sum of the purines after autolysis approximates the content of the original tissue (Experiment 1A 6.27, Experiment 1B 6.49, Experiment 1C 6.39). The behavior of the tumor tissues was quite different. In these little hypoxanthine was found after autolysis; the greater part of the purine was then present as xanthine. Presumably this represents a difference in the enzymic content of the tissues, possibly the much higher content of "xanthine oxidase" to be found in tumor tissue (8).

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· SUMMARY

Hypoxanthine (1 mg. or more of nitrogen) is determined by a volumetric method with an error of about 1 per cent. It is precipitated as the crystalline argentipicrate. This is separated from silver chloride by extraction with hot concentrated nitric acid and the silver is determined by titration with standard thiocyanate solution.

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THE INACTIVATION OF PYRIDINE NUCLEOTIDES BY ANIMAL TISSUES IN VITRO

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The disintegration of animal tissues releases a heat-labile system that rapidly destroys the biological activity of the known pyridine nucleotides (1–3). The activity of this system can be inhibited by nicotinamide (4). The products formed during the inactivation of the pyridine nucleotides are unknown. Studies have been made of the products formed by the action of other materials, *e.g.* almond meal (5), intestinal mucosa (6), and washed yeast (7), on diphosphopyridine nucleotide.

In the present work, the end-products of the inactivation of the pyridine nucleotides by animal tissues were studied.

Materials and Methods

The pyridine nucleotides were prepared from yeast (8). The standardization of the two samples of diphosphopyridine nucleotide (DPN) used has been described (9). The maximum purity of one was 0.36, of the other 0.75. The maximum purity of the triphosphopyridine nucleotide (TPN) was 0.07 based on its nicotinic acid content and V factor activity. The amounts of the nucleotides indicated in the experimental section refer to the amount of active material. Yeast adenylic acid was obtained from Hoffmann-La Roche, Inc., muscle adenylic acid from the laboratory of Dr. Parnas, and adenosine diphosphate from Dr. C. F. Cori.

Broken cell preparations of the tissues were used as a source of the enzyme system. The preparations were made by grinding tissue with sand and 0.16 M sodium chloride solution. The mixture was squeezed through muslin, and the insoluble material washed four times by centrifugation with 10 volumes of salt solution. The washed solids were suspended in 9 volumes of salt solution to which a few drops of toluene had been added. The preparation was kept in the refrigerator when not in use.

The experiments were carried out, in general, as follows: A mixture composed of 1 ml. of an aqueous solution of substrate, an appropriate volume of enzyme preparation, 1 ml. of 0.05 M phosphate buffer, pH 7.4, or

salt solution, and, where indicated, 1 ml. of an aqueous solution of other substance, was diluted to 5 ml. with water, and then incubated at room temperature. After a stated time interval, 2 ml. of 0.83 M trichloroacetic acid, pH 2.2, were added, the total volume made to 10 ml., and the mixture was then centrifuged. Aliquots of the supernatant fluid were taken for analysis. Suitable controls were prepared and treated in the same fashion as the experimental mixtures.

Pyridine nucleotides were assayed as V factor (10). The adenine nucleotides do not serve as V factor. Inorganic phosphate was determined by the method of Fiske and Subbarow (11). Nicotinamide was measured by means of its reaction with cyanogen bromide and p-methylaminophenol (12, 13) or aniline (14). The color obtained from the pyridine nucleotides in this reaction is less than 0.5 per cent as much as that obtained from an equivalent amount of nicotinic acid. The adenine nucleotides do not give this reaction. Since the determinations of the pyridine compounds were made in the presence of trichloroacetic acid, which diminishes the color produced, an appropriate amount of this acid was added to the nicotinamide and nicotinic acid standards. Under these conditions the color obtained from nicotinamide was 30 and 45 per cent as much as that obtained from an equivalent amount of nicotinic acid when p-methylaminophenol and aniline were used respectively.

<div align="center">EXPERIMENTAL</div>

Nature and Occurrence of Enzyme System—As indicated by the method of preparation, the enzyme is insoluble in water and salt solutions at pH 7.4. Drying the tissues with acetone inactivated the enzyme. All activity was destroyed in 10 minutes by heating at 70°. No appreciable loss of activity occurred when the preparation was dialyzed against running distilled water for 48 hours. As indicated by Fig. 1, optimal activity of the washed brain preparation was obtained at pH 7.5.

The enzyme inactivates both added pyridine nucleotides and that originally present in the tissues. This was demonstrated by the following experiments. Four samples of 50 mg. each of rat liver were ground in a mortar with sand and 2 ml. of water. V factor analyses were performed immediately on the first sample and on the others after standing for 30, 120, and 240 minutes respectively. Simultaneously, parallel experiments were done on rat kidney, leg muscle, intestine, and brain. After 30 minutes, 10 to 20 per cent of the original V factor was still present in each tissue preparation. After 120 minutes the V factor was present in the intestine but not in the others. After 240 minutes no V factor was present in any preparation.

, 0.2 ml. of washed preparations of the same tissues was incubated with

40 γ of DPN for 5 minutes.   V factor analyses indicated that 100, 70, 45, 40, and 20 per cent of the added DPN was inactivated by brain, liver, kidney, muscle, and intestine respectively.   The same order of activity for rat tissues has previously been reported (4).   The same order and approximately the same degree of activity were found for the tissues of the dog and rabbit.

It has been reported that reversibly reduced DPN is also inactivated by rat tissues (2).   The following experiment confirms this report.   One 50 mg. sample of rat liver was ground in the usual fashion with trichloroacetic acid solution containing ferricyanide.   The ferricyanide converts the reduced pyridine nucleotides present in the tissue to the oxidized forms
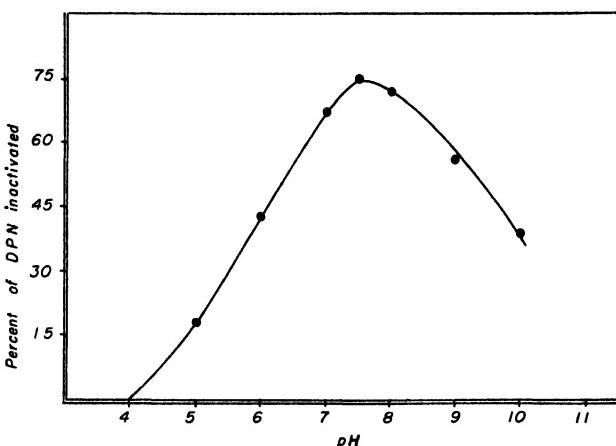
Fig. 1. The effect of pH on the enzymatic inactivation of DPN.   Each point represents the mean of four determinations of the percentage of 40 γ of DPN inactivated by 0.1 ml. of a dilute washed brain preparation incubated for 10 minutes at room temperature.

which are stable in acid solution (15).   A second sample was ground with acid alone.   In acid solution the reduced pyridine nucleotides are rapidly inactivated.   A third and fourth sample were ground with water alone. After 5 minutes the third sample was treated with trichloroacetic acid and ferricyanide, the fourth with trichloroacetic acid alone.   V factor determinations were then made on each sample.   Typical data are presented in Table I.   These data show that rat liver preparations inactivate reduced pyridine nucleotides at the same or perhaps even greater rate than the oxidized forms of these substances.

The data in Table II show that TPN as well as DPN was inactivated by washed brain preparations.   In similar experiments TPN was inactivated by rabbit liver, muscle, and kidney preparations.   This may also be in-

ferred from the probability that animal tissues contain both nucleotides and the fact that all the V factor eventually disappears from broken cell preparations of animal tissues.

Inhibition of Enzyme Activity—It has been reported that nicotinamide inhibits the inactivation of DPN by preparations of rat brain (4). This was confirmed as follows: 40 γ of DPN were incubated for 10 minutes with 0.1 ml. of washed brain preparation in the presence and absence of 80 mg.

TABLE I

Inactivation of Oxidized and Reduced Pyridine Nucleotides by Broken Cell Preparations of Rat Liver

Experimental details are given in the text. The values given are the results of V factor analyses expressed as micrograms of DPN per gm. of wet tissue.

	Liver 1	Liver 2
Initial oxidized and reduced nucleotides (1)	417	381
" " nucleotides (2).	270	290
" reduced nucleotides ((1) − (2)).	147	91
Final oxidized and reduced nucleotides (3).	90	78
" " nucleotides (4)	89	80
" reduced nucleotides ((3) − (4)).	1	−2
Oxidized nucleotide enzymatically inactivated	180	112
Reduced " " "	146	93

TABLE II

Destruction of TPN by Rabbit Brain in Presence of Nicotinamide

The figures represent the percentage of inactivation of 40 γ of TPN by a broken cell preparation of rabbit brain diluted 100 times with isotonic saline solution.

Nicotinamide	0.1 cc. enzyme		0.2 cc. enzyme	
	5 min.	1 hr.	5 min.	1 hr.
M				
0.081	9	17	12	23
0.0405	15	26	26	39
0.02	38	57	48	83
0.0000	54	89	74	98

of nicotinamide and V factor estimations then performed as usual. In the absence of nicotinamide all of the V factor disappeared. In the presence of nicotinamide less than 10 per cent disappeared. In similar experiments with washed liver, kidney, and muscle from the rat and rabbit it was found that 0.16 M nicotinamide almost completely inhibited the destruction of the nucleotide. This suggests that the activity of the different tissues may be due to the presence of the same enzyme.

The inhibitory effect of nicotinamide is quite specific. In addition to the compounds previously tested (4), picolinic acid, quinolinic acid, pyrazinemono- and dicarboxylic acids, benzamide, α-aminonicotinic acid, trigonelline, adenine, adenosine, pyridine, and nicotinuric acid were found to have no effect upon the inactivation of 40 γ of DPN by 0.1 ml. of washed rat brain preparation. The final concentration of the substances tested was 0.16 M, at which concentration nicotinamide almost completely inhibits the enzyme.

The following experiment suggests that the inhibitory effect of nicotinamide is not due to the reversal of a simple mass law equilibrium by one of the products of a reaction. 40 γ of DPN were inactivated by 0.1 ml. of washed brain preparation by incubation for 15 minutes. 80 mg. of nicotinamide were added and the incubation continued for 1 hour. The second

TABLE III

Destruction of DPN by Rabbit Brain in Presence of Nicotinamide

The figures indicated represent the percentage of inactivation of 40 γ of DPN by a broken cell preparation of rabbit brain diluted 100 times with isotonic saline solution.

Nicotinamide	0.1 cc. enzyme				0.2 cc. enzyme			
	5 min.	1 hr.	2 hrs.	5 hrs.	5 min.	1 hr.	2 hrs.	5 hrs.
M								
0.081	8	15		27	12	22		34
0.0405	11	18		34	17	26		46
0.02	17	25	37	58	33	39	45	75
0.01	21	35		63	44	49		82
0.005	38	46	58	77	56	66	80	96
0.0000	62	75	93	95	90	97	98	98

incubation was carried out in individual tests at pH 3, 4, 5, 6, 7, 7.5, and 8.0. V factor assays were then performed. No increase in pyridine nucleotides was produced by the addition of nicotinamide.

That nicotinamide does not progressively inactivate the enzyme was shown in the following manner. 0.1 ml. samples of rabbit brain preparation were incubated with 40, 20, and 10 mg. of nicotinamide in a total volume of 1.0 ml. for 1 hour. To each of these mixtures were added 40 γ of DPN and incubation was continued for 5 minutes. Samples were then taken for V factor analyses. The degree of inactivation of the DPN in each case was the same as that found when enzyme preparation was added · to the proper mixtures of substrate and inhibitor and incubated for 5 minutes.

Table III summarizes the effect of various concentrations of nicotin-

amide on a stock rabbit brain preparation which had been further diluted 10 times with salt solution. In the absence of added nicotinamide 0.2 ml. of enzyme preparation completely inactivated the substrate in about 10 minutes. At no concentration of nicotinamide employed was the destructive reaction completely inhibited and in each such case the reaction was still proceeding slowly towards completion after 5 hours of incubation. It will be seen also that 2-fold increases in the concentration of nicotinamide did not produce 2-fold increases in inhibition. These facts are compatible with the conditions described for "competitive inhibition" (16).

That nicotinamide also inhibits the inactivation of TPN is demonstrated by the data in Table II.

Products of Inactivation of DPN—Mixtures containing 500 γ of DPN (666 γ of preparation of purity 0.75), 0.4 ml. of washed rabbit brain, and 1.6 ml. of salt solution were incubated for 10, 20, 30, 60, and 120 minutes. At the times indicated 1 ml. of trichloroacetic acid solution was added, followed by 2 ml. of water. The supernatant liquids obtained by centrifugation were analyzed for V factor, inorganic phosphate, and nicotinamide.

V factor analyses indicated that all of the DPN was inactivated within 10 minutes. The color obtained in the nicotinamide analyses was equivalent to 97 per cent of the nicotinamide originally present in the nucleotide. The amount of color obtained after 120 minutes of incubation was identical with that obtained after 10 minutes of incubation. No inorganic phosphate appeared even after 120 minutes of incubation.

In a similar experiment equivalent quantities of yeast and muscle adenylic acids, adenosine diphosphate, and inorganic pyrophosphate were incubated with the brain preparation for 1 hour. In no case was either V factor or nicotinamide color found. However, inorganic phosphate was produced equivalent to 28, 48, and 36 per cent of the total phosphorus of the yeast adenylic acid, muscle adenylic acid, and adenosine diphosphate respectively. A very small amount of inorganic orthophosphate was produced from the pyrophosphate.

These experiments suggest that nicotinamide is one of the products of this inactivation. Nicotinamide was isolated from the reaction mixture, as its picrolonate, in the following manner. 75 mg. of DPN (250 mg. of 0.36 pure preparation) were incubated for 2 hours with the washed preparation from one rat brain in a total volume of 20 ml. The mixture was centrifuged and the supernatant fluid decanted. The insoluble material was washed twice in the centrifuge with 15 ml. portions of water. The supernatant fluid and washings were combined, adjusted to pH 3 with dilute sulfuric acid, and a slight excess of solid silver sulfate was added with shaking. The insoluble material was removed by centrifugation and washed twice with 10 ml. portions of water. The excess silver in the

combined supernatant fluid and washings was removed with hydrogen sulfide and the excess hydrogen sulfide was then removed by aeration. The solution was then adjusted to pH 7 with solid barium hydroxide and the precipitate which appeared was discarded. The solution was concentrated by distillation *in vacuo* to a volume of about 5 ml. and then to dryness over sulfuric acid. The dry residue was extracted five times with 10 ml. portions of absolute ethyl alcohol and the combined extracts then evaporated to about 0.5 ml. 20 mg. of picrolonic acid dissolved in 2 ml. of 50 per cent ethyl alcohol were added. This mixture was cooled in the refrigerator for 24 hours and the precipitate that formed was collected by centrifugation. Recrystallization was effected twice from a minimum of hot 50 per cent alcohol. The material melted with decomposition at 216° (uncorrected). The picrolonate prepared from an authentic sample of nicotinamide melted with decomposition at 218°. A mixture of the two preparations melted at 217°. The melting point of nicotinamide picrolonate in the literature is 217° (17). Pure nicotinic acid picrolonate melts with decomposition at 214° but the melting point of a mixture of the picrolonates of nicotinic acid and of either synthetic or isolated nicotinamide was found to be 204–208°.

Similar experiments were conducted with equivalent quantities of liver, kidney, and muscle preparations. The results were essentially the same as those described for brain, differing only in that the nicotinamide appeared and V factor activity disappeared less rapidly when these tissues were used.

The data permit at least a partial description of the products of the inactivation of DPN by these preparations of animal tissues. The generally accepted structure of DPN may be represented as, $NH_2 \overset{a}{-} OC$-pyridine $\overset{b}{-}$ ribose $\overset{c}{-}$ phosphate $\overset{d}{-}$ phosphate $\overset{e}{-}$ ribose $\overset{f}{-}$ adenine. The comparatively labile linkages and, therefore, possible points of rapid cleavage are indicated by the letters a to f.

The isolation of nicotinamide from the reaction mixture indicates that cleavage does not occur at a. The production of color equivalent to the nicotinamide content of the nucleotide, the isolation of nicotinamide from the reaction mixture, the fact that nicotinamide nucleoside can serve as V factor,[1] and the fact that known quaternary derivatives of pyridine, *e.g.* trigonelline, do not produce color in the nicotinamide assay all indicate that cleavage occurs at linkage b. Cleavages at c or d, which would produce adenosine diphosphate and adenylic acid respectively, are unlikely, since there is no production of inorganic phosphate. Further, cleavages at c or d are unlikely, since nicotinamide nucleoside can serve as V factor, nicotinamide mononucleotide might be expected to serve as V factor, and neither

[1] Schlenk, F., unpublished data.

compound would be expected to yield color in the nicotinamide analysis. In the same fashion, it can be seen that neither e nor f may be the sole site of cleavage. It appears, therefore, that only the splitting of linkage b is completely compatible with these data. The subsequent fate of the rest of the DPN molecule is unknown, except that under these conditions no inorganic phosphate is split out. It appears, then, that the immediate cause of the inactivation of DPN and TPN by animal tissues *in vitro* may be ascribed to the presence of an enzyme capable of cleaving these substances at linkage b.

<div align="center">DISCUSSION</div>

From these experiments it is apparent that the path of destruction of pyridine nucleotides by broken cell preparations of mammalian tissue is quite different from that observed with any other type of preparation. Thus, sweet almond emulsin preparations have been found to decompose DPN with the production of inorganic phosphate and of nicotinamide nucleoside (5). A sample of a nucleotidase prepared from the intestinal mucosa of a calf has been found to inactivate DPN with the formation of inorganic phosphate (6). Washed yeast preparations also inactivate DPN fairly rapidly. However, this reaction was observed to be inhibited by fructose diphosphate, glucose-6-phosphate, muscle adenylic acid, and inorganic phosphate but not by nicotinamide (7).

It cannot be stated whether the mechanism of decomposition in these broken cell preparations is that which occurs in the intact organism nor does one know whether the synthesis of DPN or TPN within the animal proceeds by a reversal of this pathway. Further, the possible metabolic significance of nicotinamide inhibition is not clear. If the concentration of this substance necessary to cause appreciable inhibition *in vivo* is as great as that necessary *in vitro*, then it is not likely that such inhibition does occur. Thus, it has been shown that in both the dog (18) and the rat (9) all of the nicotinamide of the kidney cortex is bound as V factor (DPN, TPN, nicotinamide nucleoside, and possibly nicotinamide mononucleotide) as is also more than 80 per cent of the nicotinamide of muscle and 50 per cent of the nicotinamide of liver. The maximum concentrations of free nicotinamide in these tissues would be 0.00, 0.0002, and 0.001 M for kidney, muscle, and liver respectively. Such concentrations of nicotinamide have no effect on the inactivation of pyridine nucleotides *in vitro* by these tissues.

The suggestion has been made (4) that substances other than nicotinamide might cure pellagra or blacktongue by virtue of their ability to inhibit the inactivation of cozymase. Since no substances other than those readily hydrolyzable to nicotinic acid have been found to prevent blacktongue consistently, and considering the great specificity of the nicotinamide inhibition, this does not seem very likely.

SUMMARY

The rate, inhibition, and end-products of the inactivation of pyridine nucleotides by washed, broken cell preparations of the brain, liver, kidney, and muscle of rabbits, rats, and dogs have been studied. The initial step in the inactivation of pyridine nucleotides by each of these tissues is the cleavage of nicotinamide from the remainder of the nucleotide molecule. The inhibition of this inactivation by nicotinamide has been found to be quite specific and to be probably of the competitive type.

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THE SYNTHESIS OF *ll*-S-(β-AMINO-β-CARBOXYETHYL)HOMO-CYSTEINE AND THE REPLACEMENT BY IT OF CYSTINE IN THE DIET

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In a recent communication (1) the preparation of the unsymmetrical thio ether, S-(β-amino-β-carboxyethyl)homocysteine,

$$\underset{\text{HOOC—CH—CH}_2\text{—CH}_2\text{—S—CH}_2\text{—CH—COOH}}{\overset{\displaystyle \text{NH}_2 \qquad\qquad\qquad\qquad \text{NH}_2}{\overset{\displaystyle |\qquad\qquad\qquad\qquad\quad |}{}}}$$
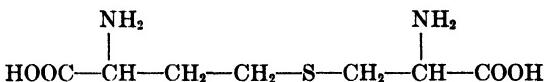
was described. Because of the possible rôle that this compound (2) or its methylsulfonium derivative (3) might play as an intermediate in the conversion of homocystine and methionine to cystine in the animal body, we were desirous of testing whether this thio ether could replace cystine in the diet. If this compound were an intermediate, it would be expected to support growth of rats on a diet free of cystine but containing an amount of methionine insufficient in itself to allow growth but which with the addition of enough cystine is capable of doing so. In order to make the metabolic experiments more conclusive we have prepared and employed the *ll* isomer, in which both asymmetric centers correspond in configuration to the naturally occurring series of amino acids. We have carried out growth studies with this *ll*-thio ether and wish to report in the present communication that this compound can support growth under these conditions.

That this compound is not cleaved to any appreciable extent to homocysteine *in vivo* was demonstrated by the fact that it was found to be incapable of supporting growth of the animals on a diet free of methionine but containing choline. Under these conditions homocysteine is able to support growth (4).

In the light of the metabolic behavior of the thio ether as reported here, the obvious inference is that the thio ether is cleaved to yield cysteine. Whether this compound is a true metabolic intermediate in the conversion of methionine or homocystine to cystine cannot yet be decided by the

experiments reported herein. The results may be regarded as supporting, but not proving, the theory of Brand, Block, Kassell, and Cahill (2) regarding the mechanism of the conversion. Further work will be required to ascertain whether the cleavage of this thio ether to cysteine, indicated by the present growth studies, is an incidental phenomenon or whether we have here a direct clue to the long sought for mechanism of the conversion of homocystine and methionine to cystine.

EXPERIMENTAL

Synthesis of ll-S-(β-Amino-β-carboxyethyl)homocysteine—The amino acid was synthesized by the methods developed for the synthesis of the racemic compound (1) and the synthesis of the meso (5) and the active (6) lanthionines. The *l*-α-amino-β-chloropropionic acid hydrochloride was prepared by the methods of Fischer, as we have previously outlined (6). The *l*-homocysteine was prepared by the reduction (7) of S-benzyl-*l*-homocysteine of $[\alpha]_D^{21} = +23°$ (8). This sample of *l*-homocysteine possessed a sulfhydryl value which was 89 per cent of the theoretical value and a portion which was oxidized yielded homocystine of $[\alpha]_D^{20} = +77°$.

15.3 gm. of *l*-homocysteine were transferred to a 500 cc. 3-necked flask. A stream of nitrogen was passed through the flask and 31 gm. of KOH in 54 cc. of oxygen-free H_2O were added. The flask was surrounded with a water bath heated to 50°, and the solution was stirred while 16.4 gm. of the *l*-α-amino-β-chloropropionic acid hydrochloride were added over a period of 1 hour. A considerable amount of KCl precipitated. After the solution had stood for 3 hours, 100 cc. of oxygen-free H_2O were added with complete solution of the precipitated KCl. The nitrogen atmosphere was maintained, and the solution was neutralized to pH 6.5 by the addition of HCl. The flask, still filled with nitrogen, was tightly stoppered, and was placed in the refrigerator overnight. The precipitate which formed contained very little homocystine and amounted to 15 gm. It was dissolved in 150 cc. of 2 N HCl, was treated with a small amount of darko at room temperature, and was filtered. The solution was slowly neutralized with 3 N NH₄OH. A total of 9.35 gm. of crystals which were largely rectangular parallelepipeds was collected. After two more such recrystallizations, 8.0 gm. of an analytically pure sample, representing 36 per cent of the theoretical yield, were obtained. The crystals darkened at 270° and decomposed at 312°.[1] A 1 per cent solution of the amino acid in 1 N HCl possessed a rotation of $[\alpha]_D^{20} = +23.7°$. The compound had the following composition.

C₇H₁₄O₄N₂S. Calculated, N 12.58, S 14.39; found, N 12.52, S 14.63

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[1] All melting points are corrected.

N,*N*′-*Dibenzoyl-ll-S-(β-amino-β-carboxyethyl)homocysteine*—250 mg. of the amino acid were dissolved in 1 cc. of 4 N NaOH and 1 cc. of benzoyl chloride and 4 cc. of 4 N NaOH were added slowly with stirring. 25 cc. of H_2O were added and the mixture was acidified with HCl. The product was filtered and was extracted twice with 20 cc. portions of benzene. The remaining material was recrystallized three times from 70 per cent alcohol

TABLE I

Data on Food Consumption

Basal diet containing	Rat No. and sex	Experimental period	Supplement to basal diet	Average daily food consumption
		days	*per cent*	*gm.*
0.20% *dl*-methionine	713 ♀	8	No supplement	3.5
		6	1.48 thio ether	5.7
		4	0.62 " "	5.7
	714 ♀	8	No supplement	4.3
		6	1.48 thio ether	5.8
		4	0.62 " "	7.2
0.4% *l*-cystine	715 ♀	19	No supplement	3.9
	716 ♀	19	" "	3.9
	717 ♀	4	" "	4.8
		14	0.40 *l*-cystine	6.2
	771 ♂	6	No supplement	5.7
		12	0.74 thio ether	8.2
	772 ♀	6	No supplement	6.5
		12	0.40 *l*-cystine	7.8
	773 ♂	19	No supplement	6.2
	715 ♀	7	" "	2.0
		11	0.74 thio ether	2.5
		7	0.46 *dl*-homocystine	5.1
	716 ♀	7	0.74 thio ether	2.6
		17	0.20 *dl*-methionine	5.6
	774 ♀	6	No supplement	2.8
		12	0.40 *l*-cystine	2.4
	775 ♂	6	No supplement	2.5
		12	0.46 *dl*-homocystine	5.6
	776 ♂	6	No supplement	2.7
		16	0.74 thio ether	3.1

and yielded 250 mg. of the derivative which melted at 229° and had the following composition,

$$C_{21}H_{22}O_6N_2S. \quad \text{Calculated, N 6.52; found, N 6.59}$$

Feeding Experiments—White rats weighing 60 to 80 gm. were given a diet in which the amino nitrogen was supplied by an amino acid mixture (free of sulfur-containing amino acids) modeled after that used

by Rose and Rice (9). The basal diet had the following composition: amino acid mixture 21.3, sucrose 44.7, Crisco 30.0, and salt mixture (Osborne and Mendel (10)) 4.0 parts respectively. The sulfur-containing amino acids were added to this diet in the amounts indicated in Table I. The fat-soluble vitamins A, D, E, and K were furnished in a corn oil solu-
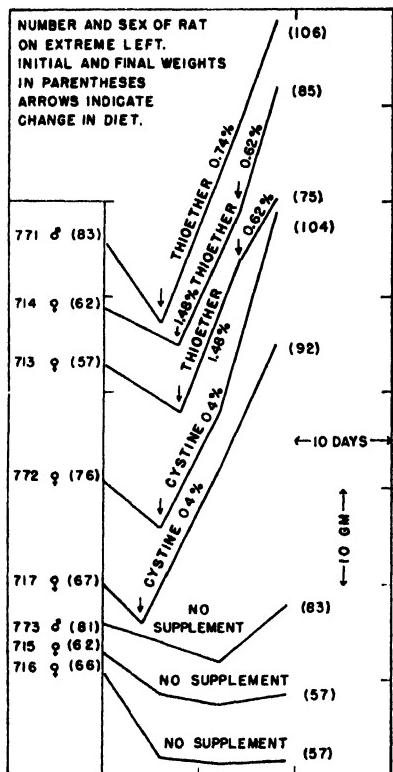
FIG. 1

FIG. 2

FIG. 1. Growth curves of rats receiving a basal diet containing 0.2 per cent *dl*-methionine, with additions of *l*-cystine or the thio ether indicated on the curves.

FIG. 2. Growth curves of rats receiving a basal diet containing 0.4 per cent *l*-cystine with additions of the thio ether, *dl*-homocystine, or *dl*-methionine as indicated on the curves.
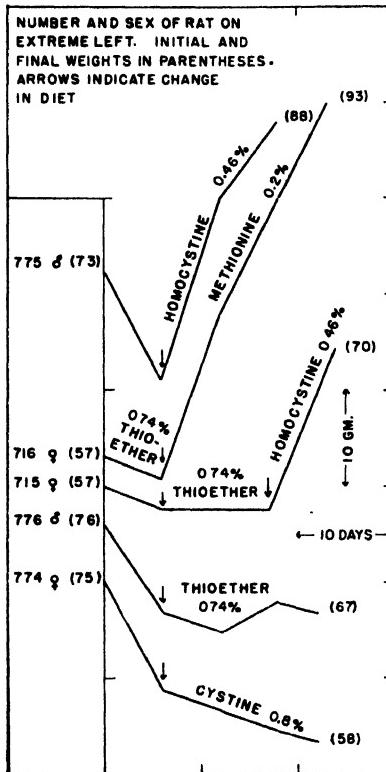
tion, 4 cc. of which were added to the fat component of 1 kilo of diet. The sources of these vitamins and the quantities supplied were the same as those described in a previous paper (11). 0.5 cc. of a water solution containing the following vitamins was fed twice daily. 1 cc. of this solution contained 20 γ of thiamine, 20 γ of riboflavin, 20 γ of nicotinic acid, 20 γ of pyridoxine, 200 γ of *dl*-calcium pantothenate, 5.0 mg. of inositol, and 25 mg. of choline

chloride. In addition to the above vitamins, Rats 771 to 776 inclusive received daily 25 mg. of ryzamin-B.

The rats used came from two litters, Rats 713 to 717 from one litter and Rats 771 to 776 from the other.

Eight rats whose growth curves are shown in Fig. 1 were given a basal diet which contained 0.2 per cent *dl*-methionine in addition to the constituents listed above. The animals failed to grow on this diet, but good growth resulted in the case of Rats 717 and 772 when 0.4 per cent cystine was also included in the diets. Rats 713, 714, and 771 grew equally well when the thio ether was added to the diet instead of cystine. It is to be noted that the 0.74 per cent level of the thio ether was equivalent in sulfur content to the 0.4 per cent level of cystine.

In a second series of experiments, for which the growth curves are shown in Fig. 2, five rats received a basal diet containing 0.4 per cent cystine, but no methionine. Growth was not possible on this diet because of the deficiency in the essential amino acid methionine. When methionine was added to the diet of Rat 716, good growth resulted. Likewise the addition of homocystine to the diet enabled Rats 715 and 775 to grow, as would be expected, since the diet contained sufficient choline to bring about the conversion of homocystine or homocysteine to methionine. In contrast to this result it is to be noted that growth did not result from the addition of the thio ether to the basal diets of Rats 715, 716, and 776, even though the amount of thio ether administered afforded as much sulfur as an effective level of methionine or homocystine.

Data on the average food consumption of rats used in these experiments are given in Table I.

SUMMARY

The synthesis of *ll*-S-(β-amino-β-carboxyethyl)homocysteine has been described.

It has been shown that this thio ether can serve in lieu of cystine in the diet for the support of growth of animals. The possible significance of this finding to the question of the mechanism of the conversion of methionine and homocystine to cysteine has been discussed.

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STUDIES ON TISSUE WATER

I. THE DETERMINATION OF BLOOD WATER BY THE DISTILLATION METHOD

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The standard method for determining the water content of biological materials is drying to constant weight in an oven, usually at 105°. The loss in weight is assumed to represent the original water content of the sample.

The absolute accuracy of this procedure depends on the validity of two assumptions, (*a*) that the attainment of constant weight implies complete removal of water, and (*b*) that the entire weight loss is due to the volatilization of water. The first assumption is subject to the criticism that material dried to constant weight at one temperature loses weight when exposed to a higher temperature (1). Neuhausen and Patrick (2) heated a silica gel to 300° *in vacuo* for 6 hours without reducing the water content below 4.8 per cent and Bartell and Almy (3) state that water persists within silica gels at temperatures well above the critical temperature of water. Rimington (4) states that 2 to 7 per cent of water adheres to protein after it has been dried to constant weight preliminary to elementary analysis. His evidence for this statement is the fact that the values for hydrogen and oxygen in proteins are too high to agree with what is known of their amino acid composition. Benedict and Manning (5) demonstrated that proteins dried under a high vacuum for several weeks actually gain weight in an oven at 110° but lose it when again dried in a high vacuum.

The validity of the second assumption, that all the weight loss in oven drying represents volatilization of water, is equally questionable. Benedict and Manning (6) demonstrated experimentally the volatilization of both fatty materials and nitrogen from proteins heated in an oven at 100–105°. This would increase the weight loss and hence yield high values for water. The magnitude of the error is (in all except adipose tissue, at least) much smaller than the opposing error due to incomplete removal of water (6), so that the net result is a value lower than the actual water content.

65

A further source of error in the determination of water by oven drying is gain in weight due to absorption of oxygen (oxidation), likewise demonstrated by Benedict and Manning (6). The errors due to volatilization of substances other than water and to oxidation are minimized by drying *in vacuo* at 20°, but several weeks may be required for the attainment of constant weight. The error due to incomplete removal of water is, of course, not diminished by this procedure; in fact it probably is increased.

Aside from the errors inherent in the drying method, the length of time required and the numerous weighings involved make it inconvenient as a routine procedure.

The determination of water by distillation with liquids immiscible with water has certain advantages which should make it a method of choice for biological work. The apparatus is inexpensive and is easily constructed by any competent glass-blower. The method is rapid, requiring 30 to 40 minutes for blood plasma or serum and 1 hour for whole blood. The removal of water is complete (see the analyses on protein solutions in this paper), there is no contact with oxygen, and, if substances other than water are volatilized, they are probably much more soluble in the organic distillation fluid than in water, so that water recovery is not measurably increased.

Marcusson (7) apparently was the first to determine moisture by distilling the sample with a liquid immiscible with water (he used xylene). Rogers (8) recommended the use of toluene. Dean and Stark (9) improved the method by providing the receiving tube with a side arm which connected to the distillation flask, allowing the reflux distillation of the toluene, while the water was trapped by dropping to the bottom of the receiving tube. The method presented in this paper is a modification of the reflux distillation method of Dean and Stark.

<center>EXPERIMENTAL</center>

Apparatus—The apparatus is shown in Fig. 1. It is constructed of Pyrex and all joints are No-lub standard taper 24/40. The 250 ml. distilling flask is flat bottomed and has a short neck formed by an outer standard taper. The condenser is the West type (jacket length 300 mm.) with a sealed-on drip-tip, which must be long enough to dip about 10 mm. beneath the surface of the toluene in the receiving tube when the apparatus is assembled. This prevents the return of water droplets to the distillation flask, decreasing the distillation time. The open portion of the tip faces the side arm. The receiving tube is made from 25 mm. tubing; to the tapered bottom is sealed a 1.00 ml. Mohr pipette, with graduation intervals of 0.01 ml. The receiving tube has a side arm, 10 mm. in diameter, which leads to the distillation flask. A mercury leveling bottle is attached by

rubber tubing to the tip of the pipette. The rubber tubing is pretreated with dilute acid and alkali to remove the bloom, and the mercury should be redistilled; both must be cleaned and dried if the mercury becomes dirty. An L-shaped glass tube, connected to the top of the condenser by a standard taper joint, dips about 10 mm. beneath the surface of toluene in an open flask. This allows free vapor expansion during distillation, while preventing the condensation of atmospheric moisture in the condenser tube. The position of the flask is adjusted so that the vertical distance between the top of the condenser and the level of toluene in the flask is about 300 mm. This permits adequate but not too rapid siphoning of toluene for rinsing the condenser at the end of an analysis (described below).
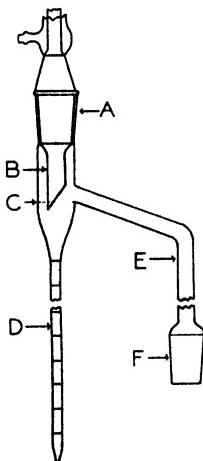
FIG. 1. Receiving tube with condenser tip in place. *A*, No-lub joint between condenser and receiving tube; *B*, drip-tip sealed to bottom of condenser joint; *C*, toluene level during distillation; *D*, Mohr pipette (1.00 ml. with graduation intervals of 0.01 ml.); *E*, side arm of receiving tube; *F*, joint to which distillation flask is attached.

Procedure

The receiving tube is attached, without lubrication, to the condenser. The mercury leveling bottle is connected to the receiving pipette, the mercury level is raised to the junction of the pipette and the receiving tube, and the rubber tubing clamped with a screw clamp. A 1.00 ml. sample of blood or plasma is delivered into a distillation flask containing 100 ml. of 4 per cent *n*-amyl alcohol in toluene. The amyl alcohol reduces the tendency toward the formation of adherent water films in the condenser and receiving tube. The joint between the distillation flask and the side arm of the receiving tube is lubricated lightly with rubber cement, which may be obtained from any shoe repair shop, and the flask is secured in place.

Toluene is delivered through the top of the condenser until it reaches the level of the side arm of the receiving tube. The glass tube connecting the top of the condenser to the open flask of toluene is adjusted as described above. With the water jacket of the condenser empty, the distillation flask is heated until the first appearance of boiling, and then the water current through the condenser jacket is turned on. This preliminary heating frees the system of all atmospheric moisture.

Distillation is allowed to proceed at a brisk rate for 1 hour for whole blood or 40 minutes for plasma or serum. At the end of this time the drop of water which usually adheres to the condenser tip is dislodged by raising the mercury cautiously until the water in the receiving tube makes contact with the drop. The mercury is now lowered until the water-toluene junction is about 10 mm. below the junction of the receiving tube and the pipette, and the heating is discontinued. As cooling proceeds, contraction of the air in the system results in the siphoning of sufficient toluene from the open flask to rinse the condenser thoroughly, dislodging any water droplets which may adhere to the walls. After the recovered water has cooled to room temperature, it is drawn into the pipette and measured. Water droplets occasionally adhere to the walls of the receiving tube. They are easily dislodged by disconnecting the condenser and scraping the walls of the receiving tube with a rubber-tipped stirring rod. After each analysis the receiving tube is cleaned by rinsings in the following order: alcohol, water, warm chromic-sulfuric acid cleaning fluid, water, alcohol. The tube is dried in a stream of dry compressed air or in an oven. The condenser is cleaned in like manner after three or four analyses.

Calibration and Correction Factors—The pipette of the receiving tube is calibrated with mercury in the usual manner before it is sealed to the tube, or it may be calibrated after sealing by measuring the mercury delivered into it from a calibrated burette. The Mohr pipettes examined have been found to *contain* from 0.995 to 1.000 ml. In addition, there is a constant volume correction characteristic of each assembly and independent of the volume of water recovered. This factor, which amounts to 0.03 ml. for an assembly with the dimensions given above, is determined by blank analyses on distilled water; this volume is added to the measured volume of water. If a substance is added to the toluene to reduce the glass-wetting tendency of water, a correction must be applied for the solubility of this substance in water. The use of 4 per cent *n*-amyl alcohol in toluene increases the apparent recovery of water by 1.0 per cent. Theoretically this factor should vary with the volume of water in the sample, since the greater the relative proportion of water, the lower the equilibrium concentration of amyl alcohol in toluene. Actually the low solubility of amyl alcohol in water and the great excess of toluene result in a very constant factor (see Table I).

Choice of Distillation Fluid—Several liquids immiscible with water have been tested. Xylene, benzene, mixtures of the two, and petroleum ether give inconstant results. Toluene, either alone or with the addition of amyl alcohol, gives constant results. Butyl alcohol is satisfactory in reducing wetting, but causes unpredictable variations in water recovery. Solid camphor has the advantage of requiring no correction, but is somewhat less efficient than amyl alcohol. Heptane (b.p. 90–100°), the only aliphatic hydrocarbon used, gives constant results (see Table I) and is superior to

TABLE I

Recovery of Known Volumes of Distilled Water

Analysis No.	Distillation fluid	Volume of sample	Volume of water recovered
		ml.	*ml.*
1	Toluene alone	1.000	1.000
2	" "	1.000	1.000
3	" "	1.000	1.000
4	" "	0.500	0.500
5	" "	0.500	0.500
6	" "	0.500	0.502
7	1 % camphor in toluene	1.000	1.000
8	1 % " " "	1.000	0.990
9	1 % " " "	1.000	0.995
10	1 % " " "	0.700	0.700
11	1 % " " "	0.700	0.710
12	4 % *n*-amyl alcohol in toluene	1.000	1.000
13	4 % " " " "	1.000	1.000
14	4 % " " " "	1.000	1.000
15	4 % " " " "	1.000	0.999
16	4 % " " " "	1.000	0.995
17	Heptane	1.000	0.990
18	"	1.000	1.000
19	"	1.000	1.000
20	"	1.000	1.003

toluene alone from the standpoint of reduction of glass-water films, but has the disadvantage of requiring a more prolonged distillation. Its lower boiling point should make it desirable for the analysis of materials high in volatile components.

Results

Analyses on Distilled Water—Table I gives the results of twenty consecutive distillations of known volumes of distilled water. The average recovery is 99.95 per cent of the theoretical, with a mean deviation of 0.26 per cent.

Analyses on Protein Solutions—The data presented in Table I indicate that water in the free form is quantitatively distilled and measured. It remained to be demonstrated that water is completely extracted from protein solutions by distillation with toluene. Since it is impossible to obtain proteins in dry form (4) (samples of powdered egg albumin tested contained 12 to 15 per cent water), solutions of egg albumin (Merck's impalpable powder) were made in distilled water. The solutions were allowed to stand overnight in the cold, and then centrifuged four times (1 hour at 3000 R.P.M.). The protein content was determined by Kjeldahl analysis (N × 6.25), and specific gravity was determined by the falling drop method of Barbour and Hamilton (10). For a given sample, the weight of water is equal to the total weight minus the weight of protein. No distinction is made between "free" and "bound" water in the analyses of protein solutions, blood, and plasma.

The results of a typical experiment are as follows: protein content, 9.79 gm. per 100 ml.; specific gravity, 1.03374 at 27.0°; water content (calculated), 90.5 gm. per 100 gm.; water content by distillation, 90.3 gm. per 100 gm.; water content by oven drying,[1] 89.0 gm. per 100 gm.

Analyses on Blood and Plasma—In order to eliminate individual variations, all analyses have been made on blood from a single dog, an adult, male mongrel, weighing 26.2 kilos. Blood samples were drawn into oiled syringes from the femoral artery and transferred to flasks containing sufficient ammonium and potassium oxalate to give a final concentration of 2 mg. of oxalate per ml. of blood. Since the water content of whole blood depends primarily on the relative proportion of cells and plasma, the water content of the plasma also was determined. The water content of the cells was calculated by the formula given below. Hematocrit cell volume was determined by centrifugation (3000 R.P.M. for 1 hour) under oil in Wintrobe tubes. All determinations were made in quadruplicate. Plasma was obtained by centrifugation under oil (to prevent evaporation). Hemolyzed samples were discarded. The specific gravity of whole blood and plasma was determined by the falling drop method (10). 1 ml. samples of blood and plasma were delivered into distillation flasks from an Ostwald-Van Slyke blood pipette which had been repeatedly calibrated by weighing samples of blood and plasma delivered. A delivery time of 45 to 60 seconds is essential for complete drainage of the pipette. Flat, glass-stoppered drying vessels were used for water determination by the oven drying method. At 105° plasma samples attained constant weight in 24 hours; whole blood required 36 to 48 hours.

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[1] Constant weight was attained after 5 days in the oven at 105°. No further loss of weight occurred after 22 days in the oven.

The specific gravity of the cells was calculated by the formula

$$SG_c = \frac{100SG_b - V_p SG_p}{V_c}$$

where SG_c = specific gravity of cells, SG_b = specific gravity of blood, SG_p = specific gravity of plasma, V_c = hematocrit cell volume, V_p = plasma volume $(100 - V_c)$.

The water content of the cells was calculated by the formula

$$W_c = \frac{100W_b SG_b - V_p W_p SG_p}{V_c SG_c}$$

where W_c = water content of cells (by weight) and W_b and W_p = water content of whole blood and plasma respectively.

TABLE II

Comparison of Results by Distillation and by Oven Drying

The results are measured in gm. per 100 gm.

Sample No.	Blood water			Plasma water			Cell water (calculated)*		
	Distilla-tion	Oven drying	Differ-ence	Distilla-tion	Oven drying	Differ-ence	Distilla-tion	Oven drying	Differ-ence
1	79.5	76.7	2.8	93.2	91.1	2.1	67.3	63.6	3.7
2	81.3	79.2	2.1	92.6	91.5	1.1	68.9	65.5	3.4
3	79.5	77.3	2.2	92.9	91.1	1.8	66.8	64.3	2.5
4	82.5	80.1	2.4	94.1	92.3	1.8	68.1	65.0	3.1
5	82.1	80.1	2.0	93.5	92.1	1.4	68.2	64.4	3.8
6	74.0†	73.5†	0.5†	93.0	91.6	1.4	67.6	67.0†	0.6†
7	83.7†	82.6†	1.1†	93.0	91.6	1.4	68.9	66.1†	2.8†
Averages .	81.0	78.7	2.3	93.2	91.6	1.6	68.0	64.6	3.3

* See the text.

† Omitted from the averages for the reasons given in the text.

The results of duplicate determinations on five samples of blood obtained on different days from the same animal are shown in Table II. Samples 6 and 7 were prepared from Sample 5 by centrifuging and resuspending the cells in plasma. Since the relative proportion of cells and plasma in Samples 6 and 7 was purposely altered, the values for the water content of whole blood are not included in the averages. The differences in Samples 6 and 7 between values for whole blood water by the drying method and by distillation are excluded from the averages because, due to a faulty thermostat, the oven temperature reached 150°. The apparent increased water yield in these two samples (as compared to the distillation values) may be due to actual volatilization of more water at the higher temperature, or to partial pyrolysis with liberation of volatile components.

The values for water content of whole blood, plasma, and cells by the oven drying method are in good agreement with those in the literature (11). The values obtained by the distillation method are uniformly higher. In the case of plasma, the average difference is almost identical with that observed in the analyses on egg albumin solutions. The discrepancy is greater in the case of cells, as might be expected from their higher protein content and greater structural complexity.

In order to determine the reproducibility of results with the distillation method, ten analyses were performed on a single sample of blood (Sample 4 of Table II). The results, in order of determination, were 82.4, 82.6, 82.5, 82.3, 82.3, 82.5, 82.4, 82.5, 82.3, 82.5 gm. per 100 gm. These results, together with those on distilled water and on protein solutions, indicate an average maximum deviation between duplicate determinations of 0.2 per cent.

Preliminary investigations have been made into the cause of the low results obtained with the oven method. Two 10 gm. samples of blood were dried to constant weight in the oven. The dried residue from one sample was transferred, with minimal exposure to the atmosphere, to a distillation flask and distilled for 2 hours. The water recovered accounted for 20 per cent of the difference between the results by oven drying and by distillation. The dried residue from the other sample was ground under toluene in a mortar and then transferred to a distillation flask and distilled for 2 hours. The water recovered in this case accounted for 65 per cent of the difference between oven drying and distillation values. These results are best interpreted on the assumption that small amounts of water are trapped in pockets in the heat-denatured protein during oven drying. Mechanical grinding of the dried residue breaks down the walls of these pockets and exposes the residual water to the action of the toluene. When fresh samples of blood are distilled, the extraction of water is complete before heat denaturation occurs. Toluene denaturation apparently does not result in the trapping of water, since blood samples preserved under toluene for 24 hours yield the same results as samples freshly distilled.

SUMMARY

A method is described for the determination of water in blood by distillation with toluene. The analysis requires 1 hour and has a reproducibility of 0.2 per cent. The results are uniformly higher than by the oven drying method (2.3 per cent for whole blood, 1.6 per cent for plasma, and 3.3 per cent for cells (calculated)). The sources of error in the oven drying method are discussed, and reasons are given for believing that lower results by this method are due to incomplete extraction of water.

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THE OPTICAL ROTATION OF *l*-CYSTINE

A Correction

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The writer is indebted to Professor H. B. Lewis for calling his attention to a misleading error in the article, "The specific rotation of *l*-cystine in relation to degree of neutralization and pH," by Toennies, Lavine, and Bennett.[1]   While the data shown in Fig. 1 of that paper were obtained with mercury light, $[\alpha]_{Hg}$, as is evident from the text, the measurements shown on p. 496 were obtained with sodium light; so that in lines 2, 3, and 5 of that page $[\alpha]_D^{31\ 5}$ should be substituted for $[\alpha]_{Hg}^{31\ 5}$.

[1] Toennies, G., Lavine, T. F., and Bennett, M. A., *J. Biol. Chem.*, **112**, 493 (1935–36).

THE PREPARATION OF HISTIDINE BY MEANS OF 3,4-DI-CHLOROBENZENESULFONIC ACID

By HUBERT BRADFORD VICKERY

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Doherty, Stein, and Bergmann (1) have recently described a series of aromatic sulfonic acids which form relatively insoluble salts with certain of the amino acids. These substances were examined with the object of determining their possible value as reagents for determinations of amino acids in proteins by the solubility product method (2). The table of solubilities they give shows that three of the acids[1] studied yield salts (disulfonates) with histidine sufficiently insoluble to offer the possibility that these substances might be used as reagents for the direct precipitation of histidine from hydrolysates of suitable proteins. A study has accordingly been made of the conditions under which the most readily available of these sulfonic acids may be employed for the preparation of histidine in quantity. It is hoped to extend the investigation to the development of an analytical method for histidine.

When from 5 to 6 M proportions of 3,4-dichlorobenzenesulfonic acid, calculated from the quantity of histidine assumed to be present, are added to a concentrated solution of the products of hydrolysis of coagulated red blood cells (crude hemoglobin), histidine slowly separates, in the cold, in the form of a dense mass of prismatic crystals of the disulfonate.[2] This salt is readily purified by recrystallization from hot water, the use of an excess of the reagent in the mother liquor being necessary to repress the solubility only in the last of successive crops of crystals. The substance is easily characterized by its high decomposition point (about 280°) which differs widely from the much lower true melting points of the salts the reagent forms with leucine, phenylalanine, and arginine, the chief contaminants to be anticipated under the conditions described. Histidine can be recovered

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[1] 3,4-Dichlorobenzenesulfonic acid, O-(2,4-dinitrophenyl)-*p*-phenolsulfonic acid, and 2,6-diiodophenol-4-sulfonic acid.

[2] In the lack of a convenient short name for 3,4-dichlorobenzenesulfonic acid, it has been necessary to refer to the salts of this acid discussed in the present paper by the general term sulfonates.

by removal of the sulfonic acid as its barium salt, and isolated either as the free base or as the monohydrochloride. The yields are high and the final product is satisfactorily pure.

The chief difficulty with the procedure is the tendency of leucine monosulfonate to crystallize from the hydrolysate along with the histidine disulfonate. Although the leucine salt is far more soluble than that of histidine, so much leucine is present in blood cell hydrolysates that substantial quantities at times separate in the form of radiating masses of needles. Fortunately, however, the leucine salt, in the impure form in which it is encountered, has an extraordinary capacity to form supersaturated solutions. By recrystallization of the mixture under the correct conditions, histidine can be brought to separate nearly completely, and it is possible to decant most of the mother liquor from the histidine disulfonate crystals before separation of the leucine salt occurs. Purification can then be carried out without difficulty.

The advantages of a method to prepare histidine by the direct crystallization of a salt over the methods in current use that involve the precipitation of complex compounds with silver (3) or mercury (4) scarcely need emphasis. Although the yield is doubtless smaller than can be secured by careful application of the nearly quantitative silver precipitation technique, the time and labor required are far less.

EXPERIMENTAL

3,4-Dichlorobenzenesulfonic Acid—125 ml. (163.5 gm.) of *o*-dichlorobenzene (Eastman, declared purity 95 per cent) and 270 ml. of concentrated sulfuric acid are heated in an oil bath for 24 hours at 185° ± 5° under an air condenser. The mixture is cooled and poured into 1500 ml. of water, decolorized with norit, and concentrated *in vacuo* to about 800 ml. The solution is cooled and stirred during the rapid crystallization. After being chilled for a few hours, the acid is filtered on a sintered glass funnel, or on a layer of drilling or canvas press-cloth in a Buchner funnel, dilution being made with concentrated hydrochloric acid as necessary to facilitate transfer. The white crystals are washed free from sulfuric acid with concentrated hydrochloric acid, sucked as dry as possible, and, if time is not a factor, may be transferred to a shallow dish and stored over calcium oxide, preferably *in vacuo*, until dry and free from chloride ion. This may require several weeks.

Alternatively, the moist crystals are transferred to a weighed beaker and are fused and heated with a small flame until the hydrochloric acid and much of the water have been expelled. Absence of chloride ion can usually be demonstrated when a temperature of 130° has been reached. The product is cooled and weighed to obtain the crude yield, which is usually from 225 to 235 gm., is again fused (70°), and 250 ml. of chloroform are

slowly added with stirring. Two turbid liquid layers form, and the mixture is stirred, while being cooled, in order to obtain the acid in small crystals. After being chilled for a few hours, the product is filtered, washed once with cold chloroform, and exposed to a warm dry atmosphere until free from solvent; yield, approximately 83 per cent of theory. Such preparations usually retain a small excess of water and yield two turbid phases when dissolved in hot chloroform.

Preparations of the free acid obtained after long continued drying over calcium oxide appear to contain 2 molecules of water of crystallization and yield a clear solution in hot chloroform. A part of the water is stable under usual dehydrating conditions. When heated for a few hours at 105°, the crystals lost only 4 to 5 per cent (theory for $2H_2O$, 13.68 per cent) in weight, and only 7.8 per cent was removed in 2 hours at 140°. A sample purified by crystallization from hot chloroform contained 12.3 per cent of sulfur (theory for $C_6H_4O_3SCl_2 \cdot 2H_2O$, 12.18 per cent), but after having stood over calcium chloride for a week, contained 12.8 per cent of sulfur and 28.6 per cent of chlorine (theory 26.95). The atomic ratio of chlorine to sulfur was 2.02, but about one-third of the water of crystallization had been removed by the calcium chloride. The presence of water of crystallization was established qualitatively by distilling a small sample at 140° into a cold tube that contained anhydrous copper sulfate; the blue hydrate was at once formed.

Preparations of the acid dried over calcium oxide melt at about 67°, occasionally as high as 69°; when cooled, they solidify promptly and melt again, when heated, about a degree higher than the original melting point. After recrystallization from hot chloroform, the hydrated substance melts at 71–72°.

The method of sulfonation described is the simplest found to be satisfactory, little attention being required if an electric heater is employed. The product usually contains a trace of insoluble impurity which can be removed by filtration of a concentrated aqueous solution of the reagent if desired, but this is not necessary if it is to be used for preparation work.

As an alternative method of purification, the sulfonation mixture may be poured into 20 per cent sodium chloride solution, the sodium salt which separates being then decolorized with norit and recrystallized from salt solution. It is converted into the barium salt dihydrate by the addition of an equivalent of barium chloride to a hot aqueous solution, and the free acid is liberated by treatment of a suspension of the barium salt in hot water with an equivalent of sulfuric acid. The barium sulfate is removed, the solution is concentrated, and the acid is crystallized by the addition of a liberal amount of concentrated hydrochloric acid and obtained in dry form as already described.

Hydrolysis of Protein—The appreciably higher yields of histidine that

can be obtained from material relatively rich in hemoglobin render it well worth while to prepare coagulated red blood cells as described in a previous paper (3). For part of the present work, two preparations made from carefully washed horse blood red cells, for which we are deeply indebted to Professor E. J. Cohn, were used; in addition, commercial "technical hemoglobin" (Eastman) was studied.[3]

Of the air-dry crude protein, 500 gm. are thoroughly mixed with 1000 ml. of water and 1500 ml. of concentrated hydrochloric acid are added; the suspension is heated on the steam bath for several hours until it has become notably less viscous and is then boiled under a reflux for 24 hours. The excess of acid is removed by concentrating the hydrolysate *in vacuo* to a thick syrup three times successively, after the addition of water. The dark solution is diluted to about 4 liters, boiled with sufficient norit to reduce the color to pale yellow, and filtered; the norit is extracted twice with boiling water after removal from the filter, and the solution and washings are concentrated to somewhat less than 2 liters. The loss of nitrogen during decolorization is of the order of 2 to 3 per cent, and the final solution should be at a reaction between pH 1.2 and 1.6.

Precipitation of Histidine—225 gm. of 3,4-dichlorobenzenesulfonic acid are added in dry form and the volume is adjusted to approximately 2 liters. The solution is chilled and it is advantageous to add seed crystals of histidine disulfonate, made in advance from a *pure* preparation of histidine or one of its salts. Crystallization of the histidine salt should begin promptly but no agitation, beyond an occasional swirling, should be attempted. After 3 days in the cold, the histidine will have crystallized practically completely as a dense mass of stout prisms; if the solution has become filled with thin needles arranged in radiating masses, separation of leucine monosulfonate in addition has occurred. The subsequent steps depend upon which of these two conditions is encountered. If there has been no obvious crystallization of the leucine compound, the solution is carefully but rapidly *decanted* from the mass of crystals as completely as possible, about 1 liter of a cold 4 per cent solution of the reagent is at once added to the sludge of crystals, and these are transferred to a filter, and sucked dry. Although the main solution of the hydrolysate is heavily supersaturated with leucine monosulfonate, it does not usually deposit the salt so rapidly as to interfere with this operation.

If, on the other hand, the greater part of the main solution is filled with crystals, the entire mass is transferred to a filter, and washed with a cold

[3] Preparation 1 of blood cells, after being extracted with alcohol and dried in air, contained 15.69 per cent nitrogen, 4.95 per cent moisture, and 1.70 per cent ash; Preparation 2 contained 15.52 per cent nitrogen, 4.74 per cent moisture, and 1.71 per cent ash. The "technical hemoglobin" as received contained 14.82 per cent nitrogen, 7.43 per cent moisture, and 2.97 per cent ash.

4 per cent solution of the reagent. The crystals are dissolved in about 1 liter of boiling water, boiled with a little norit, filtered, and the volume is adjusted to about 1300 ml. The solution is chilled and seeded with pure histidine disulfonate; crystallization of the histidine salt is rapid and is usually complete after 3 to 4 hours in the refrigerator. The entire success of the preparation depends upon the correct handling of this solution; it is heavily supersaturated with leucine monosulfonate, and crystallization may occur with dramatic suddenness if it stands too long or is unduly agitated or becomes contaminated with seed crystals of this salt. Furthermore, the relative proportions of histidine and leucine salts present may differ in different preparations, with corresponding differences in sensitivity to influences that start the crystallization of the leucine salt.

The clear supernatant solution is carefully but rapidly poured away from the crystals into another beaker. Nearly all of the solution can usually be decanted, although it frequently sets solid at once, and some crystallization may occur even in the stream of solution as it runs away from the histidine disulfonate crystals. If the separation is found to be impossible, or is only partially successful, more water is added and the suspension of crystals is heated until all is again in solution. The operations are repeated at successively greater dilutions until a satisfactory decantation is achieved, but sufficient time must always be allowed for a complete crystallization of the histidine salt.

Purification of Histidine Disulfonate—The crude salt, obtained as described, is dissolved in boiling water, and treated again with norit. The volume from which the crystallization is allowed to take place depends upon the purity with respect to leucine. About 500 ml. of boiling water are sufficient to recrystallize 100 gm. of histidine disulfonate if relatively pure, but at least twice as much should be used if substantial amounts of leucine salt are still present. The crystals are washed with a little cold 4 per cent solution of the reagent. After being recrystallized twice, the purified product is dried *in vacuo* over sulfuric acid, since sufficient reagent adheres to the crystals to darken them if dried in the oven at 105°. The trace of reagent is then removed by washing the dry crystals with ether. On being heated, the compound darkens from about 270° and decomposes with evolution of gas at about 279–280° (short thermometer, uncorrected). Table I gives data on the composition of the main crops of material from a number of preparations.

Small additional quantities of the histidine salt can be secured from the combined mother and wash liquors from the recrystallization by evaporation *in vacuo*. However, the possibility that leucine may be present must always be kept in mind, and application of the decantation technique to separate this is sometimes necessary.

The yield of histidine disulfonate depends upon several factors, among

the less obvious ones being apparently the relative rates at which the histidine and leucine salts separate from the hydrolysate. In cases in which crystallization of the leucine salt began early, the quantity of histidine isolated was depressed, doubtless because of removal in the leucine sulfonate of much of the desired excess of reagent. In such cases, the main mother liquor, after having been concentrated to the original volume, was treated with an additional quantity of reagent, usually about 75 gm., and was returned to the refrigerator for a few days. The crop of mixed histidine and leucine sulfonates that separated was treated as already described, and more histidine salt was readily secured. Table I shows, in the column headed "Second crop," the quantities of additional histidine salt thus obtained. Clearly, since the behavior in any specific case cannot be anticipated, it is good practice always to treat the main mother liquor in this way if high yields are desired.

TABLE I

Preparation of Histidine from Coagulated Blood Cells and from Technical Hemoglobin

Material	N content H_2O-free	Quantity H_2O-free	Histidine disulfonate					Yield of histidine	
			First crop	Decomposition point	N content*	Second crop			
	per cent	*gm.*	*gm.*	*°C.*	*per cent*	*gm.*		*gm.*	*per cent*
Blood cells	16.51	472.5	121.8	278	6.88			31.03	6.57
	16.51	424.6	108.6	276–277	6.88			27.68	6.52
	16.29	474.9	91.54	279–281	6.87	14.29		26.95	5.68
Technical	16.02	461.5	83.6	275–276				21.31	4.62
hemoglobin	16.02	462.4	58.36	277–279		23.71		20.91	4.52

* Theory for $C_6H_9O_2N_3 \cdot (C_6H_4O_3SCl_2)_2$, 6.90 per cent.

*Crude Leucine Monosulfonate—*Considerable effort has been expended upon systematic fractional recrystallizations of the large masses of impure leucine monosulfonate that separated during several of the preparations in the hope that a little more histidine could be secured, and also to obtain information regarding their composition. Aside from direct evidence, from the ready formation of the unmistakable monoflavianate, that a little arginine was present in some of the intermediate fractions,[4] and from qualitative tests that suggested the presence of traces of phenylalanine in others, little was learned save that leucine is the main component. However, repeated recrystallization of such material seldom led to a product of fixed and sharp melting point, and after decomposition of the presumably purified material and fractional crystallization of the free amino acids,

[4] One small fraction contained 19 per cent of arginine, instead of the theoretical 27.7 per cent for arginine disulfonate, but most contained only traces.

only the first crop as a rule possessed the nitrogen content of pure leucine, subsequent crops being successively lower in nitrogen.

A pure specimen of leucine monosulfonate, made from authentic material, melted at 194–195°, a preparation of the phenylalanine salt at 196–197°, and the well crystallized and moderately insoluble arginine disulfonate at 205°; all three solidify when cooled a few degrees and melt within a degree of the original melting point when heated again. The various crops of crude leucine monosulfonate secured during the fractionation of material derived from the hydrolysates as a rule melted over ranges of several degrees at temperatures well below 195°. In a few cases, small amounts of histidine disulfonate were indeed obtained, but it became evident that fractionation for this purpose was not worth while. Furthermore, little encouragement was found for the view that the crude monosulfonate might become a useful source of pure leucine. On the other hand, however, the separation of the monoamino acid salts from the main mother liquors proved to be the simplest method to recover the bulk of the reagent from them if this were desired, since a pure preparation of the barium salt of the sulfonic acid can readily be obtained if the salts are decomposed as described in a subsequent paragraph.

The order of magnitude of the relative quantities of histidine and of crude leucine sulfonates encountered may be inferred from incidental data secured during the third experiment reported in Table I. In this case, 91.5 gm. of histidine disulfonate were separated from the first crop by decantation and subsequent recrystallization; the decanted solution yielded a number of successive crops of crude leucine salt weighing in all 124 gm. From the second treatment of the main mother liquor, 14.3 gm. of histidine salt and 60 gm. of crude leucine salt were likewise secured. These figures neglect appreciable quantities of leucine salt present in the final mother liquors obtained in working over the material.

Preparation of Histidine—As an example of the procedure for the recovery of histidine from the disulfonate, the details of a single experiment will be given: 108.1 gm. of recrystallized histidine disulfonate (equivalent to 27.54 gm. of histidine) were dissolved in 800 ml. of boiling water, and cold saturated barium hydroxide solution was added until the reaction was at approximately pH 7.2 as shown by brom-thymol blue. This required about 800 ml. After being chilled for a few hours, the well crystallized barium sulfonate dihydrate was filtered off and washed free from histidine (color test with reagents of Koessler and Hanke (5)) with cold water. This was set aside for recovery of the reagent. The filtrate was concentrated *in vacuo* to 600 ml., and, in order to remove as much sulfonic acid as possible, was brought to about pH 8.5 (faintly alkaline to phenolphthalein paper) with barium hydroxide, and chilled for a few hours.

The small additional precipitate of barium salt was removed and the clear filtrate was exactly freed from barium with sulfuric acid. The barium sulfate was centrifuged and thoroughly washed, and the clear solution (reaction close to pH 7.2) was concentrated *in vacuo* until a copious crystallization of histidine had occurred. The solution (about 250 ml.) was heated at atmospheric pressure until all crystals had dissolved, and an equal volume of alcohol was added. After being chilled overnight, 20.69 gm. of free histidine were obtained; decomposition point about 272°, nitrogen 27.1 per cent, theory for $C_6H_9O_2N_3$, 27.10 per cent.[5]

The filtrate was concentrated to 100 ml. and, since it had become acid, was neutralized to pH 7.4 with ammonia, filtered from a trace of turbidity, and treated with 2 volumes of alcohol. A second crop of 5.09 gm. of histidine separated. The mother liquor was found to contain a little sulfonic acid, owing to the solubility of barium sulfonate. It was accordingly concentrated to remove alcohol and the small remaining amount of histidine was recovered, after acidification to Congo red with hydrochloric acid, by the addition of a liberal excess of the sulfonic acid reagent; 5.72 gm. of the disulfonate separated, equivalent to 1.45 gm. of histidine (1 gm. of disulfonate contains 0.2548 gm. of histidine). The total recovery was thus nearly 99 per cent. In another experiment in which 164.8 gm. of the disulfonate were worked up (equivalent to 41.99 gm. of histidine), the successive crops of histidine weighed, respectively, 32.63 and 7.84 gm., and the disulfonate from the final mother liquor, 5.46 gm., equivalent to 1.39 gm. of histidine; in all 99.7 per cent of the histidine taken.

Histidine prepared in this way, although satisfactory with respect to decomposition temperature and nitrogen content for all ordinary uses, is not entirely pure when rigid criteria of purity are employed. Several lots amounting to 94 gm. were combined and recrystallized once from aqueous alcohol, whereby 86.3 gm. of decomposition point 270° and nitrogen content 27.1 per cent were obtained in the first crop. The preparation was found to contain 0.13 per cent of ash, and the mother liquor, when concentrated, deposited a trace of inorganic impurity soluble only in hydrofluoric acid; apparently a little silica had been acquired from the glass. A specimen of this material was kindly examined by Professor M. S. Dunn in comparison with a specimen prepared and extensively purified by other methods in his laboratory. The specific rotation was $-38.80°$ ($t = 25.0°$, $c = 2.67, l = 4$ dm., $\lambda = 5893$ Å.), while that for the undissolved part, when a sample was equilibrated at 25° with enough water to dissolve about half of it, was $-38.70°$; Dunn's sample gave $-38.70°$ and $-38.75°$ respectively under similar conditions, all specific rotations being measured with a pre-

[5] The nitrogen of histidine can be completely recovered by the macro-Kjeldahl method only if digestion is continued for 6 to 8 hours after the solution becomes colorless.

cision of ±0.07°. Tests of this kind are capable of demonstrating the presence of less than 0.4 per cent of *dl*-histidine and the material was clearly pure by this criterion.

The solubility at 25.0°, determined gravimetrically, was 0.04414 ± 0.00011 gm. per ml.; Dunn's sample, under the same conditions, had a solubility of 0.04156 ± 0.00006 gm. per ml., indicating a slight impurity in our sample as compared with his. Furthermore, a standard decomposition time curve (6) was found to lie about 3° lower on the temperature scale over the range 270–293° examined, again suggesting the presence of a minor degree of impurity in our sample.

Preparation of Histidine Monohydrochloride Monohydrate—104.4 gm. of histidine disulfonate were treated as described in the previous section up to the point at which the barium sulfate had been removed from the solution of free histidine. To the clear solution, which was at pH 7.2, was added 1 mole of hydrochloric acid (6.36 gm.) calculated for the histidine present; the resulting solution was now at pH 2.8.[6] The solution was concentrated *in vacuo* to approximately 100 ml., and 200 ml. of hot alcohol were added; crystallization began promptly and, after the solution had been chilled overnight, 32.5 gm. of lustrous plates of the monohydrochloride monohydrate separated, the equivalent of 90.2 per cent of the histidine present. The mother liquor was concentrated to 100 ml. and treated with an excess of the sulfonic acid reagent; 8.16 gm. of histidine disulfonate were recovered, equivalent to an additional 7.8 per cent.

The histidine monohydrochloride monohydrate was dried at 105°; it contained 19.99 per cent nitrogen (theory for $C_6H_9O_2N_3 \cdot HCl \cdot H_2O$ 20.04 per cent) and lost 8.9 per cent in weight when further dried at 135° for 4 hours (theory 8.59 per cent). No ash could be detected.

DISCUSSION

The salt of histidine with 2 moles of 3,4-dichlorobenzenesulfonic acid is unusual in many ways. Its capacity to crystallize in nearly pure form even from the mixture of amino acids in a concentrated solution of the products of hydrolysis of crude hemoglobin distinguishes its behavior sharply from that of such compounds as the flavianates, picrates, or picrolonates of histidine, or from the salts with mineral acids. There is a close analogy with the behavior of the flavianates of arginine (8), but among compounds of histidine only the complexes formed with silver or mercury salts have hitherto been found useful for the initial separation from the hydrolysate; these are formed only at neutral or alkaline reactions.

The fundamental difference appears to lie in the fact that histidine di-

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[6] This was slightly too acid, probably due to the presence of a little sulfonic acid arising from the solubility of barium sulfonate. A reaction in the range pH 3 to 4 is desirable ((7) p. 834).

sulfonate is least soluble at reactions so strongly acid that amino acids are completely dissociated as bases; under these circumstances effects on the solubility due to the presence of dipolar ions are at a minimum.   There are few types of organic reagents, other than sulfonic acids, with which this region of acidity can be conveniently reached, and Bergmann's work with these substances is clearly pointing the way to a new understanding of the behavior of the salts of amino acids.   It is a curious fact that the possibilities of using ordinary *mineral* acids to precipitate amino acids from protein hydrolysates should have been practically exhausted at the first attempt[7] when Hlasiwetz and Habermann (10) in 1873 showed that glutamic acid hydrochloride could be crystallized directly from a sufficiently acid solution.   Kossel's introduction in 1924 of flavianic acid (11) as a reagent for arginine was the first fundamental advance since that time and opened a field of investigation that is only now being explored.

3,4-Dichlorobenzenesulfonic acid as a reagent for histidine differs sharply from flavianic acid (12), picric acid (13), and picrolonic acid (14) in that there is no tendency under any ordinary circumstances for the formation of the monosulfonate to occur; this appears to be a very soluble compound, little success having attended efforts to prepare it from free histidine and 1 mole of the sulfonic acid.   The disulfonate does not separate under these conditions, since the presence of sufficient acid to bring about the dissociation of the second basic group is essential; in practice this can be provided either as hydrochloric acid or as a liberal excess of the reagent. Careful adjustment of the reaction is not necessary as it is in the preparation of the mononitranilate (15).

The most serious disadvantage of the present procedure, as a method to prepare histidine from blood cells, is the tendency of leucine monosulfonate to separate in an impure form from the hydrolysate along with the histidine compound.   The artifice by which the histidine salt is isolated is undeniably one that calls for experience, although, once the technique has been learned, it gives little difficulty.

Arginine disulfonate appears only as a minor contaminant of the leucine salt when one is working with hemoglobin hydrolysates, but with a protein of high arginine content, such as edestin, arginine disulfonate separates in considerable amounts.   There is no likelihood of contamination with lysine, however, in any case, since the lysine disulfonate is a very soluble compound.

SUMMARY

When a liberal excess of 3,4-dichlorobenzenesulfonic acid is added to a decolorized hydrochloric acid hydrolysate of crude hemoglobin at pH 1.2

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[7] See, however, the interesting experiments of Barnett (9) on the salting-out of dileucine hydrochloride.

to 1.6, histidine slowly separates in the cold as the relatively insoluble di-3,4-dichlorobenzenesulfonate. The histidine salt is at times accompanied by a considerable quantity of impure leucine mono-3,4-dichlorobenzene-sulfonate, but the mixture can readily be separated by recrystallizing and taking advantage of the marked capacity of the impure leucine salt to form a supersaturated solution. Most of this can be removed from the crystals of histidine salt by decantation under the correct conditions; the histidine salt is then readily purified by recrystallization. Histidine is recovered in satisfactorily pure form, either as free base or as the monohydrochloride, after removal of the sulfonic acid as its insoluble barium salt. The yields depend upon the purity of the starting material with respect to its content of hemoglobin; from fairly pure preparations of red blood cells, the equivalent of over 6 per cent of histidine has been obtained.

It is a pleasure to acknowledge the help of Professor M. S. Dunn and of Professor A. White in checking certain details, as well as Dr. M. Bergmann's courteous encouragement to undertake the investigation.

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COMPLEXES OF DEHYDROASCORBIC ACID WITH THREE SULFHYDRYL COMPOUNDS*

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The formation of addition compounds by the interaction of carbonyl and sulfhydryl groups was observed as early as 1885 by Baumann (1) who described addition compounds of phenyl mercaptan with chloral, pyruvic acid, benzoyl formic acid, and isatin. Later investigations (2–5) have confirmed the initial work of Baumann and have greatly extended the knowledge of carbonyl-sulfhydryl addition compounds. In more recent studies Schubert (6) reported the isolation of addition compounds (1:1) formed from phenyl- and methylglyoxals by reaction with glutathione, thioglycolic acid, and thioglycolic acid anilide. These compounds were characterized as 1-hydroxyalkylthio ethers, while compounds of cysteine and thiourea with the glyoxals were found to be substituted thiazolidines (7). Schubert (8) has also isolated crystalline addition compounds of cysteine with six reducing sugars (aldoses), but a comparable product from cysteine and fructose was not isolated. In aqueous solutions these compounds and the cysteine aldehyde compounds absorbed iodine as rapidly as free cysteine and slowly deposited free cystine. A thiazolidine structure was suggested for the compounds on the basis of their similarity to the cysteine aldehyde compounds which had been shown to be thiazolidines (7, 9). Ågren (10) has also recently reported the formation, in neutral aqueous solutions, of cysteine-aldose compounds to which he assigned a thiazole structure.

Since the early work of Szent-Györgyi (11) on "hexuronic acid," it has been known that sulfhydryl compounds can reduce dehydroascorbic acid. It is clear that dehydroascorbic acid can be utilized efficiently by guinea pigs as a source of ascorbic acid, and numerous investigations, primarily

for the purpose of testing theories concerning the chemical rôle of the vitamin, have been carried out on the reduction of dehydroascorbic acid *in vivo* and *in vitro* (12). The most pertinent of these in relation to the present study is the investigation of Borsook *et al.* (13). These workers suggested that an intermediate compound was probably formed when dehydroascorbic acid was reduced by glutathione. The suggestion was based primarily on the finding that approximately the same percentage of dehydroascorbic acid was reduced by glutathione in 15 to 30 minutes when the glutathione-dehydroascorbic acid ratio was varied from 100:1 to 4:1. In addition, the rate of reduction was too rapid to be accounted for by a third order reaction. The latter, of course, need not imply the formation of a complex with a measurable lifetime. The present investigation was undertaken to obtain more conclusive evidence regarding the formation of complexes from dehydroascorbic acid and sulfhydryl compounds and to determine the ratio in which the reactants combine.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Reagents*</div>

Dehydroascorbic Acid—Crystalline ascorbic acid (0.3 gm.) was dissolved in 5 ml. of molar acetic acid. To this solution, 0.23 gm. (24 per cent excess) of steam-distilled benzoquinone was added, and the oxidation was allowed to proceed for 90 minutes at room temperature. Extraction of the solution with four 35 ml. portions of ethyl ether removed the excess benzoquinone and the hydroquinone formed during the reaction. Each ether extract was extracted with 0.5 ml. of molar acetic acid to keep the loss of dehydroascorbic acid into the ether layer at a minimum. The resulting acetic acid solution was placed in a desiccator and evacuated twice to 15 to 20 mm. pressure to remove dissolved ether. The optical rotation of the oxidized solution was determined after dilution to 10 ml. with molar acetic acid. The concentration of dehydroascorbic acid was calculated from the value $[\alpha]_D = +55°$ reported by Haworth and Hirst (14). The yield of dehydroascorbic acid was usually from 0.24 to 0.25 gm.; so the solutions were approximately 0.14 M. The solutions were diluted to the desired molarity with molar acetic acid. Titration of the solutions with iodine or 2,6-dichlorobenzenoneindophenol showed that the content of reducing substances (ascorbic acid or hydroquinone) was not greater than 2 per cent of the dehydroascorbic acid as determined from the rotation. Solutions of dehydroascorbic acid that were reduced with hydrogen sulfide at pH 3.5 and freed of excess hydrogen sulfide by nitrogen and evacuation showed, by titration with 2,6-dichlorobenzenoneindophenol, concentrations that never differed by more than 2 per cent from the concentrations determined

by rotation. The rotations of diluted solutions also checked the calculated rotations within 2 per cent.

Glutathione—Some glutathione preparations were isolated from yeast by the method of Schroeder *et al.* (15); these preparations were practically colorless and melted at 189–193°. Titration of several preparations with iodine showed a purity of 97.4 to 99.6 per cent. A preparation of glutathione from the Schwarz Laboratories, New York, was used for the titration studies; this glutathione was colorless, odorless, and pure (100 ± 0.1 per cent) as judged by titration with iodine. The solutions of glutathione were prepared by dissolving a weighed quantity of the substance in 3 or 4 ml. of water contained in a 10 ml. volumetric flask, followed by the addition of 5 ml. of 2 M acetic acid and dilution to the mark with water.

Thioglycolic Acid—Technical grade thioglycolic acid was redistilled at 15 mm. pressure in an atmosphere of nitrogen. The fraction boiling at 109–110° was immediately diluted with distilled water until solutions 1.5 to 2.0 M were obtained; these were placed in the refrigerator at 5°. The molarity was determined by titration with iodine in 1 M acetic acid solution and by titration with sodium hydroxide in aqueous solution. The two values agreed to within 0.2 per cent. The final solutions of thioglycolic acid were prepared by diluting the required quantity of the stock solution with 2 M acetic acid and water in the manner described for the preparation of glutathione solutions.

Cysteine—The calculated quantity of cysteine hydrochloride for 10 ml. of a solution of the desired molarity was weighed to 0.1 mg. and dissolved in 5 ml. of 2 M acetic acid contained in a 10 ml. volumetric flask. The free hydrochloric acid was neutralized by addition of the necessary amount of normal sodium hydroxide before the mixture was diluted to the mark with distilled water.

Rotation Studies—Since dehydroascorbic acid is optically active, a complex containing this substance and a sulfhydryl compound would probably be optically active. However, if the sulfhydryl compound were also optically active, *e.g.* glutathione or cysteine, several possibilities would need to be considered in picturing the effect of complex formation on changes in rotation. *l*-Cysteine,[1] $[\alpha]_D^{26} = +7.6°$ (16), has been shown (13) to reduce dehydroascorbic acid, $[\alpha]_D = +55°$, with the formation of ascorbic acid, $[\alpha]_D = +23°$, and cystine, $[\alpha]_D = -200°$. A mixture of the first two compounds would then show a decrease in rotation as the

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[1] The cysteine solutions used showed specific rotations of +4° to −9°, depending on the source and state of oxidation. To show that *l*-cysteine and not *dl*-cysteine was being used, a portion of the cystine formed by oxidation with dehydroascorbic acid was isolated and the rotation determined. A 1 per cent solution in 1.0 M HCl showed an $[\alpha]_D$ of −184° at room temperature (approximately 26°).

reaction proceeded, because of the lower rotation of ascorbic acid and the strongly levorotatory power of cystine. However, if a dextrorotatory complex with a specific rotation greater than that of dehydroascorbic acid were formed, an initial increase in rotation should be observed, followed by a decrease in rotation caused by the oxidation-reduction reaction. A levorotatory complex of these compounds might not be distinguished in this manner, because the oxidation-reduction reaction might mask the decreased rotation. The rotation of a mixture of glutathione,[2] $[\alpha]_D = -20°$, and dehydroascorbic acid should be analogous to the cysteine-dehydroascorbic acid mixture, since oxidized glutathione is strongly levorotatory, $[\alpha]_{5461} = -108°$ (17), and the reduced form is oxidized somewhat by dehydroascorbic acid. The use of an optically inactive sulfhydryl compound such as thioglycolic acid would lead to a somewhat simpler situation. A dextrorotatory complex could then be observed easily, while the specific rotation of a levorotatory complex would have to be of such a value that the observed rotation would be lower than could be accounted for by reduction of the dehydroascorbic acid. To test these possibilities experiments were run in the following manner.

The solutions prepared as described were brought to the temperature of tap water or to 10°, mixed, and placed in a jacketed 2.000 dm. polariscope tube kept at a temperature constant to ±1° by circulating water. Control tubes containing the individual reactants at the same dilution and temperature as they occurred in the mixture were run, from which the initial rotation could be calculated. The tube containing the mixture was placed in the polariscope and read at 1 minute intervals for 30 minutes, following which it was read at intervals of 2 to 5 minutes. With a sodium vapor lamp as a monochromatic light source, the polariscope could be read to 0.01° and the average of five readings gave values that were reproducible to ±0.01° to 0.02°, whereas single readings might differ by double that value. The rapid changes in rotation of the mixture necessitated single readings, thus accounting for the observed deviations in Figs. 1 and 2. At the conclusion of each experiment (i.e., after 1 to 2 hours), the controls usually showed no change or a change of only 0.01°, although occasionally a decrease of 0.06° to 0.08° in the rotation of dehydroascorbic acid was observed for concentrations of 0.05 M and a tube length of 2.000 dm. The results of these experiments are shown in Figs. 1 and 2. The observed increases in rotation beyond the additive values of the components were +0.38°, +0.35°, and +0.51° for the 1:1, 2:1, and 4:1 mixtures as shown in Curves A, B, and C of Fig. 1. With glutathione the increases were much less, although very definite, the values being +0.09°, +0.15°, and +0.18° respectively for the 1:1, 2:1, and 4:1 mixtures, as shown in Fig. 2. In the

⌐ [2] Glutathione, Schwarz Laboratories Bulletin No. 100, July, 1941.

case of thioglycolic acid, however, a decrease in rotation was observed, reaching a value much lower than could be accounted for by reduction. These deviations from the calculated rotation were $-0.93°$, $-1.16°$, and $-1.23°$ for the different mixtures (Curves D, E, and F, Fig. 1).
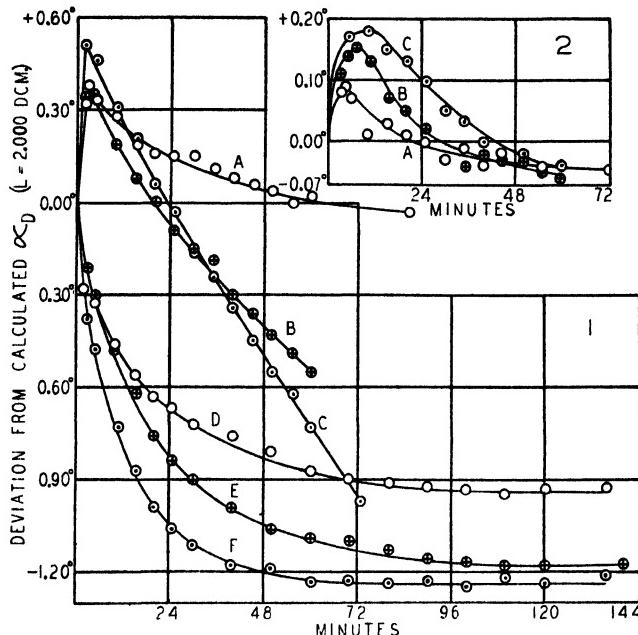
FIG. 1. Deviation of observed rotation from the calculated rotation (no reaction) for mixtures of dehydroascorbic acid and cysteine or thioglycolic acid. Curves A, B, and C are for cysteine + dehydroascorbic acid. The dehydroascorbic acid concentration was 0.06 M for each; the cysteine concentrations were 0.06 M, 0.12 M, and 0.24 M, and the temperatures were 25°, 26°, and 22° respectively. Curves D, E, and F are for thioglycolic acid + dehydroascorbic acid. The dehydroascorbic acid concentration was 0.05 M; the thioglycolic acid, 0.05 M, 0.10 M, and 0.20 M respectively, and the temperature, 10°. The solvent for all determinations was 1.0 M acetic acid. The points on Curves B, D, E, and F represent the averages of two determinations.

FIG. 2. Deviation of observed rotation from the calculated rotation (no reaction) for mixtures of dehydroascorbic acid and glutathione. The ordinates are the same as in Fig. 1. The determinations were made in 1.0 M acetic acid solutions at a temperature of 10°. Curves A, B, and C are for 0.05 M dehydroascorbic acid in the presence of 0.05 M, 0.10 M, and 0.20 M glutathione respectively.

Titration Studies—Jowett and Quastel (18) found that treating glutathione with methylglyoxal for 4 hours at pH 3.0 rendered 48 per cent of the glutathione non-titratable with iodine. Yamazoye (19) reported that the compound of methylglyoxal and glutathione, formed by mixing the two reactants, was decomposed slowly during titration with iodine in acid

solution, although the decomposition was rapid in the presence of excess iodine.   Hence, it was thought that the complexes of dehydroascorbic acid with sulfhydryl compounds, indicated by the rotational experiments, might be stable enough not to react rapidly with iodine, and that titration of a mixture of the two would require less iodine than that required for the oxidation of the sulfhydryl compound alone.   Completed oxidation-reduction between the two components would not influence the titration, since ascorbic acid would be formed, equivalent to the quantity of sulfhydryl compound oxidized.

Molar acetic acid solutions of the sulfhydryl compounds (0.10 M, 0.20 M, or 0.40 M) and of dehydroascorbic acid (0.10 M) were cooled to 0°.   Equal volumes of the solutions were mixed and replaced in an ice bath.   Samples of 1.00 or 0.50 ml. were removed at various time intervals and transferred to 10 ml. of ice-cold molar acetic acid to facilitate titration and to prevent a large rise in temperature during the titration.   The solutions were immediately and rapidly titrated with 0.01 or 0.02 N iodine, depending on the concentration of the sulfhydryl compound.   The time required for the titration was 20 to 30 seconds.   An initial end-point was obtained at which the blue color of the starch-iodine complex pervaded the entire solution and required 10 to 50 seconds to fade, depending on how far the initial end-point had been overstepped.   Further additions of iodine reestablished the slowly fading end-point until a quantity of iodine had been added sufficient for the complete oxidation of the sulfhydryl compound and the reducing substances in the original dehydroascorbic acid solution.   The end-point then remained stable.   The difference between the initial and final end-points gave a measure of the complex concentration.   The data from these experiments are shown in Fig. 3.   Curves A and B show that 54 and 37.5 per cent, respectively, of the glutathione was bound when the mole ratios to dehydroascorbic acid were 1:1 and 2:1.   Curves C, D, and E show binding of the sulfhydryl groups to the extent of 70, 44, and 22.5 per cent, respectively, for thioglycolic acid when the mole ratios to dehydroascorbic acid were 1:1, 2:1, and 4:1.

The addition of cadmium acetate to a thioglycolic acid-dehydroascorbic acid mixture in which 58 per cent of the sulfhydryl compound was bound showed that 95 per cent of the total thioglycolic acid was contained in the precipitated cadmium-sulfhydryl complex as judged by titration of the precipitate with iodine.   An iodine titration of the solution after removal of the cadmium-sulfhydryl complex by centrifugation showed that approximately 5 per cent of the reducing substances remained in solution.   Control tests on the reaction of thioglycolic acid with cadmium acetate in the absence of dehydroascorbic acid indicated that 3 to 4 per cent of the sulfhydryl groups remained in the supernatant liquid; hence the quantity

of thioglycolic acid oxidized by the dehydroascorbic acid in mixtures of the two was less than 2 per cent.

The iodine titration of mixtures of cysteine and dehydroascorbic acid in molar acetic acid solutions was complicated by the fact that in a 1 M acetic acid solution cysteine is oxidized beyond the disulfide stage and gives a poor end-point. A calibration curve was therefore prepared by
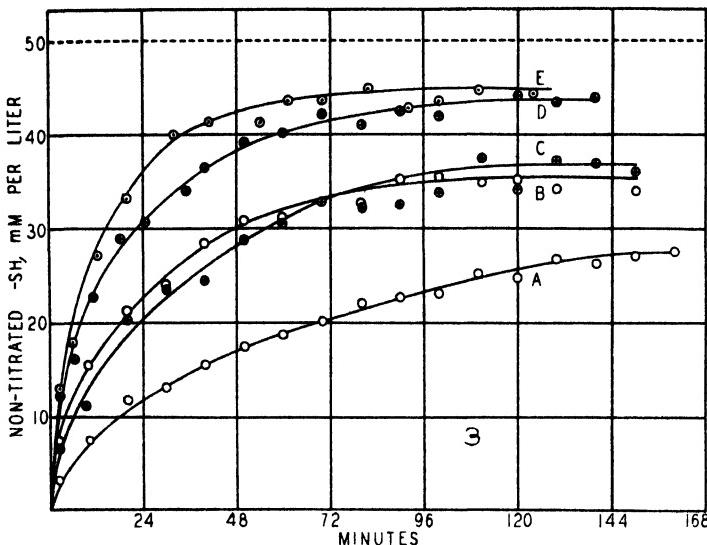
FIG. 3. Disappearance of iodine-titratable sulfhydryl groups with time in mixtures of dehydroascorbic acid with glutathione or thioglycolic acid. The dehydroascorbic acid was 0.05 M, the solvent, 1.0 M acetic acid, and the temperature, 0° for all determinations. Curves A and B are for glutathione + dehydroascorbic acid mixtures in which the former was 0.05 M and 0.10 M respectively. Curves C, D, and E are for thioglycolic acid + dehydroascorbic acid mixtures in which the concentration of thioglycolic acid was 0.05 M, 0.10 M, and 0.20 M respectively. Curves B and E are based upon the average values of two determinations; Curves C and D represent the average values from three determinations; and points on Curve A are based upon the averages of four determinations. The broken line indicates the calculated value for complete binding of the dehydroascorbic acid in a 1:1 complex.

titrating known amounts of cysteine in ice-cold molar acetic acid to the first blue color of the iodine-starch complex. Such a curve was linear over the concentration range studied and could be used for determination of the quantity of residual free cysteine in a mixture of dehydroascorbic acid and cysteine. The reproducibility of these titrations was not good, but qualitatively a complex was found to exist, because 20 to 30 per cent of the cysteine did not react during the titrations. Determinations on the

mixture were made in the same manner in which the calibration curve had been prepared.

Composition of Complex—The "method of continuous variations" as developed by Job (20) and Vosburgh and Cooper (21) was applied to determine the ratio in which the reactants combined to form the complex. Briefly the method may be described as follows:

Consider the formation of a complex to follow the general equation

$$A + nB \rightleftharpoons AB_n$$

in which A and B are the reactants and n the coefficient corresponding to the moles of B adding to one of A to produce the complex AB_n. The
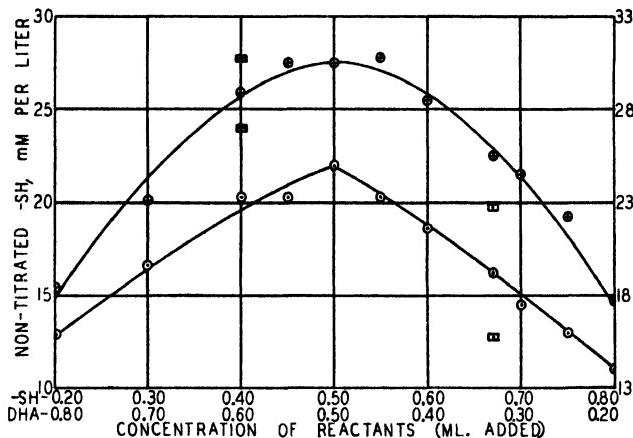
FIG. 4. Determinations of non-titrated sulfhydryl groups for various mixtures of dehydroascorbic acid with glutathione or thioglycolic acid. ⊙, average of three separate determinations when 0.10 M solutions of glutathione and dehydroascorbic acid were mixed in the indicated proportions; the ordinate scale is at the right. ⊕, average of two determinations when 0.092 M solutions of thioglycolic acid and dehydroascorbic acid were mixed as shown; the ordinate scale is at the left. ▫ and ⊞ represent the greatest extremes of the values averaged to determine a single point on the curves. The average deviation is only half of the maximum variation shown. All determinations were made at a temperature of 0°, with 1.0 M acetic acid as solvent.

determination of n may then be accomplished by mixing varying proportions of equimolecular solutions of A and B and measuring some property of the solution that is dependent on the complex concentration. When the values of the measured property (y) are plotted against composition (x), a maximum or a minimum results. The position of the maximum or minimum bears a simple relationship to n. Thus if equimolecular solutions of A and B are used and x liters of B are mixed with $(1 - x)$ liters of A, and x is varied from 0 to 1, we have $n = x/(1 - x)$ (21). The plotted curve

then yields a maximum when $x = 0.5$ if the complex is 1:1, and a maximum when $x = 0.67$ if the combination is 2:1, etc.

Equimolecular solutions of dehydroascorbic acid and the sulfhydryl compound in 1 M acetic acid were prepared and cooled to $0°$. $(1 - x)$ ml. of dehydroascorbic acid and x ml. of the sulfhydryl compound were pipetted into a reaction flask, mixed, and replaced in the ice bath. Successive samples were prepared at 7 minute intervals in which x was varied from 0.20 to 0.80; $i.e.$, the ratio of dehydroascorbic acid to the sulfhydryl compound varied from 4:1 to 1:4. Apparent equilibrium was attained at the end of 110 minutes for the thioglycolic acid-dehydroascorbic acid mixtures and at the end of 150 minutes in the case of the glutathione-dehydroascorbic acid complex. When equilibrium had been established, 10 ml. of ice-cold molar acetic acid were added to the flask, and the mixtures were rapidly titrated with iodine as previously described. The curves in Fig. 4 were obtained by plotting x against the difference between the initial and final end-points as a measure of complex concentration. Both figures show a definite maximum at $x = 0.50$, indicating clearly that the complex must be 1:1.

DISCUSSION

Curves D, E, and F in Fig. 1 show the formation of a levorotatory thioglycolic acid-dehydroascorbic acid complex and Curves C, D, and E in Fig. 3 show the extent, as measured by iodine titration, to which the complex persists in solution. The data for cadmium acetate precipitations of thioglycolic acid from reaction mixtures of the latter with dehydroascorbic acid showed that the extent of the oxidation-reduction reaction was negligible; so a combination of the two types of data should permit calculation of the specific rotation of the complex. The values found for $[\alpha]_D$ were $-14°$, $-16°$, and $-15°$ for the 1:1, 2:1, and 4:1 mixtures of thioglycolic acid and dehydroascorbic acid respectively.

Since 1:1 complexes were shown to exist, the corresponding percentages of dehydroascorbic acid bound, under the experimental conditions shown in Curves C, D, and E of Fig. 3, were 70, 88, and 90 per cent for the 1:1, 2:1, and 4:1 mixtures of thioglycolic acid and dehydroascorbic acid. Similarly, from Curves A and B in Fig. 3, values of 54 and 75 per cent are obtained for bound dehydroascorbic acid in the 1:1 and 2:1 glutathione-dehydroascorbic acid mixtures. Equilibrium constants calculated from these data deviated 2-fold for the different concentrations. However, the data fitted a mole-to-mole addition complex better than any other ratio. Equilibrium constants calculated from concentrations derived from the continuous variation curves, Fig. 4, likewise yielded values which showed 2-fold deviations. The average values obtained from both calculations are

K_D = 0.028 ± 0.005 and 0.012 ± 0.002 for the glutathione- and thio-glycolic acid-dehydroascorbic acid complexes respectively.

Calculations of the rate of formation constant of the complexes from the data in Fig. 3, considering the reaction to be second order opposed by a pseudo first order reaction, also yielded values which varied approximately 2-fold. Similar calculations based on a second order reaction opposed by one of the same order gave results similar to those obtained by the first set of calculations; so the results do not permit one to determine which mechanism was in operation.

SUMMARY

Mixtures of dehydroascorbic acid with each of three sulfhydryl compounds, glutathione, cysteine, and thioglycolic acid, were studied in acetic acid solutions. The data presented on the changes in optical activity and on the quantity of iodine required for titration of the mixtures show clearly that the two components in each mixture reacted to form a complex or addition compound.

The method of continuous variations demonstrated that the reactants combined in equimolecular quantities. Equilibrium constants calculated from the data were consistent with the mole-to-mole ratio for the complexes.

Equilibrium constants for the glutathione-dehydroascorbic acid and the thioglycolic acid-dehydroascorbic acid complexes are reported.

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THE HOMOGENEITY OF BUSHY STUNT VIRUS PROTEIN AS DETERMINED BY THE ULTRACENTRIFUGE*

By MAX A. LAUFFER

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Since the isolation of a protein possessing tobacco mosaic virus activity from diseased tobacco plants (6), viruses have been considered by some to be protein molecules. If a definition is accepted which requires that all of the molecules of a given substance be composed of the same kind and number of atoms arranged in the same way, one of the minimum conditions a virus protein preparation must satisfy in order to be considered molecular in nature is absolute homogeneity with respect to size and shape. As was early pointed out by Svedberg and his collaborators (9), the ultracentrifuge may be very useful for the determination of the degree of homogeneity of protein preparations.

When a protein solution is centrifuged under ideal conditions at high speed, the particles or molecules move towards the periphery of the centrifuge at a more or less uniform rate. The protein particles which were originally at the top of the column of solution form a boundary between protein solution and solvent, a boundary which sediments at the rate of the protein particles. If the centrifugation process is ideal, such boundaries become more and more diffuse as sedimentation proceeds. This is always due in part to the diffusion of the protein but may also be due in part to inhomogeneity. To demonstrate homogeneity by means of the ultracentrifuge, it is necessary to show that the observed boundary spreading can be accounted for entirely by the known diffusion rate of the material.

Eriksson-Quensel and Svedberg (2) were the first to study purified tobacco mosaic virus in the ultracentrifuge. They showed that the observed boundary spreading of one of the early samples prepared by chemical means by Stanley was entirely too great to be accounted for by diffusion on the assumption of any reasonable value for the rate of diffusion. Wyckoff (10) later studied the sedimentation of somewhat more favorable preparations of the virus isolated by differential centrifugation and con-

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cluded, by qualitative inspection of the sharpness of the sedimenting boundaries, that the virus was homogeneous. However, pictures of tobacco mosaic virus particles obtained more recently with the electron microscope almost always have shown that there is a distribution of particle lengths about a mean value (8). Although preparation of the mounts for the electron microscope may cause some breakage of the particles, it seems likely that qualitative inspection of the sharpness of a sedimenting boundary cannot be regarded as a satisfactory criterion of particle homogeneity.

Tomato bushy stunt virus protein, first isolated and crystallized by Bawden and Pirie (1), was early shown to consist of particles either spherical or nearly spherical in shape. Sedimentation equilibrium studies carried out by McFarlane and Kekwick (4) indicated that the distribution of the virus particles in a centrifugal field of about 150 g after a long period of centrifugation was approximately that which one would expect of a homogeneous material. This is a reasonable criterion of homogeneity but not a particularly sensitive one. Sedimentation velocity measurements at much higher centrifugal fields were also made by these workers, and it was observed at the qualitative level that the sedimenting boundaries were very sharp, again a reasonable though not critical indication of homogeneity.

In view of the facts (a) that no virus has heretofore been subjected to really critical physical tests for inhomogeneity without yielding evidence of inhomogeneity, (b) that bushy stunt virus is known not to possess a sufficient degree of inhomogeneity to be detected by less sensitive criteria, and (c) that the particles of this virus are approximately spherical, thereby making of it a favorable medium for a critical test, it was thought worth while to subject this material to the most exacting physical test for inhomogeneity possible with existing ultracentrifugation techniques. To that end, the spreading of a bushy stunt virus boundary in an ultracentrifugation experiment was measured very carefully and then was compared with the theoretical spreading calculated from the known diffusion constant of the virus on the assumption of absolute homogeneity.

EXPERIMENTAL

A sample of bushy stunt virus prepared by Dr. W. M. Stanley (7) was dissolved in 0.1 M phosphate buffer at pH 7 at a concentration of 3 mg. per ml. The solution was introduced into a 6 mm. sector-shaped centrifuge cell and spun in a Bauer and Pickels type air-driven ultracentrifuge (9) for 4 hours at 9000 R.P.M. The distance of the axis of rotation from the center of the cell was 65 mm. The temperature before and after the run was 20.9°. Photographs of the sedimenting boundary were taken at regular intervals according to the Lamm scale method (9). Various values of

scale distance were used. An exact duplicate run was made with the cell filled with buffer alone, and this served to define the base-lines for the scale diagrams.

Five of the scale exposures were chosen for analysis, and boundary diagrams of the conventional type were plotted out according to the general method outlined in Part III, B of Svedberg and Pedersen (9). The five comparable exposures on the buffer control run were used for determining the base-lines. The areas under the five curves were measured with a planimeter and were then reduced to a sort of standard state by correcting for scale distance and position of the center of the boundary with respect to the meniscus. The variation of these corrected areas about their mean was random, indicating that the scale-cell distance was fairly accurately known and also that there was no appreciable amount of material separating from the main component. The measured average area was 10 per cent greater than the value calculated from the refraction increment given by McFarlane and Kekwick (4), 0.00164, and the concentration of the virus, 3 mg. per cc., measured by the Kjeldahl method.

The theoretical boundary spreading was computed, in general, according to the method outlined in the section of Svedberg and Pedersen (9) just cited. The diffusion constant taken for the virus was that measured by Neurath and Cooper (5) by the Lamm method, 1.15×10^{-7}, corrected to water at $20°$. It was assumed that diffusion began when the centrifuge attained its running speed of 9000 R.P.M. Since it took only 2 minutes for the acceleration of the machine and since the times of diffusion of the exposures studied varied from 50 to 220 minutes, no appreciable error could result from the possible incorrectness of this assumption. The curves were so computed that each one would have a corrected area equal to the average of the corrected areas measured for the actual boundary diagrams.

<center>DISCUSSION</center>

In Fig. 1 are presented the results of the investigation. As abscissas are plotted distances from the axis of rotation in cm. and, as ordinates, scale line displacements in μ. Each curve represents the position and degree of sharpness of the sedimenting boundary at a definite time after the beginning of sedimentation. The distance from the scale to the center of the cell, which is a measure of the magnification of the ordinate, varied from diagram to diagram. Both the times and the cell-scale distances for the various diagrams are indicated in the description of Fig. 1. The open circles are the experimental data describing the actual boundary spreading and the smooth curves represent the sharpness the boundaries ought to have if spreading were due solely to the diffusion of a strictly homogeneous material, calculated as outlined in the preceding paragraph.

The excellent agreement indicates that, contrary to an earlier opinion (3), the bushy stunt virus particles are indeed exceedingly homogeneous with respect to sedimentation rate and, therefore, with respect to size, shape, and density.

An attempt was made to evaluate somewhat more critically the meaning of the agreement here obtained between theoretical and observed boundary spreading. For the purpose of doing this, bushy stunt virus was visualized as being composed of a family of particles with a distribution of sedimentation rates obeying the normal frequency distribution law. Theoretical curves for the boundary after 220 minutes of sedimentation were then calculated for the cases in which the standard deviations of the distributions were 2 per cent and 5 per cent of the mean rate.[1] These theoretical boundary diagrams and the one computed on the assumption of absolute homogeneity are shown in Fig. 2. The actual boundary data, the same as those on the last curve of Fig. 1, are also presented. It may be seen that it is a little difficult to decide with certainty between the theoretical boundary diagrams calculated for absolute homogeneity and for a 2 per cent spread, but there can be no doubt that the one for a spread of 5 per cent is eliminated. Actually the data do favor the boundary computed for the case of absolute homogeneity. It can be concluded, then, that these experiments have shown that bushy stunt virus particles either are absolutely homogeneous with respect to sedimentation rate or are represented by a sedimentation rate distribution with a standard deviation no greater than 2 per cent of the mean. The physical meaning of this conclusion is clearer if this hypothetical variation in sedimentation rate is ascribed to a

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[1] This problem was attacked in a non-rigorous manner, as follows: The sedimentation and diffusion processes were visualized as taking place independently, in sequence. First, the boundary was pictured as moving from the meniscus to its final position in practically no time. After this the boundary should be represented approximately by a normal curve with a standard deviation equal to σ/S times the distance the boundary moved, where S and σ are the mean and the standard deviation of the sedimentation rates. Since the standard deviation of a normal curve is equal to $\sqrt{2Dt}$, where t is time and D is the diffusion coefficient of the material whose boundary is represented by that normal curve, one can calculate a time, t_0, which it would have taken for the boundary to acquire its current state of sharpness by a process of diffusion. The diffusion process was next visualized as taking place for a time t equal to that of the experiment, while the boundary remained stationary. The final boundary diagram should have approximately the shape it would have had if diffusion had proceeded for a time $t + t_0$. In this process the same corrections were applied to D that are used in obtaining the theoretical diagram for the case of a strictly homogeneous material. To make the calculations as simple as possible, it was assumed that a small variability in the sedimentation constant will not cause a measurable change in the diffusion rate, an assumption justified by the relative insensitivity of the diffusion process to small changes in particle size and shape.

variation in the radius of the particles. A 2 per cent variation in sedimenta-
tion rate would correspond to a 2 per cent variation in the square of the
radius or to about a 1 per cent variation in the radius and in the diameter.
Hence, these data may be reinterpreted somewhat more loosely to mean
that bushy stunt virus particles either are of identical size or are repre-
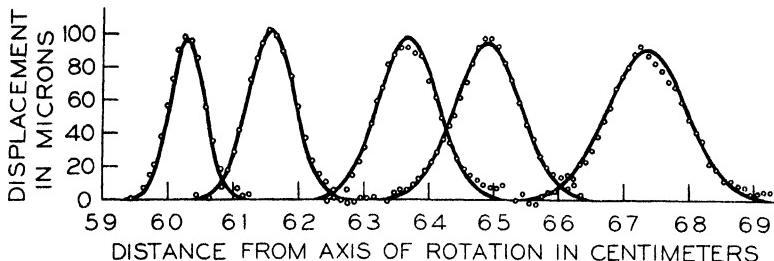
Fig. 1. Theoretical *versus* actual boundary spreading in sedimentation experiment
on bushy stunt virus. The circles are experimental points obtained by the Lamm
scale method. The smooth curves are theoretical boundary diagrams calculated
from the known diffusion constant. The times and the scale-cell distances for the
successive boundary curves, beginning at the left, are 50 minutes, 2.1 cm.; 85 minutes,
3.1 cm.; 135 minutes, 4.1 cm.; 150 minutes, 4.6 cm.; 220 minutes, 5.6 cm.
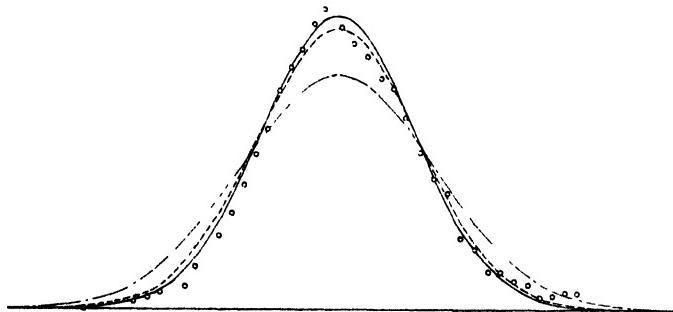
Fig. 2. Theoretical boundary curves computed on the assumption that the sedi-
mentation rate of the bushy stunt virus particles may be represented by normal
curves with standard deviations of 0 per cent (solid line), 2 per cent (dash line), and
5 per cent (dot and dash line) of the mean sedimentation rate, compared to the
actual boundary diagram obtained after 220 minutes of centrifugation (circles).

sented by a distribution of diameters with a standard deviation no greater
than 1 per cent of the mean diameter. It is unlikely that very many of
the proteins generally conceded to be in a molecular state of dispersion
have been demonstrated to be homogeneous within limits any narrower
than this. Therefore, the case for believing that bushy stunt virus protein
particles are molecules is as good as for any protein.

SUMMARY

The spreading of a tomato bushy stunt virus boundary during a sedimentation velocity experiment was measured by the Lamm scale method and was compared to the theoretical spreading one should expect due to the known diffusion rate of the material if the virus is strictly homogeneous. The results showed that the boundary spreading can be accounted for satisfactorily in terms of diffusion. A more detailed consideration of the data indicated that the method as here applied was sufficiently sensitive to exclude the possibility of the virus particles being represented by a normal size distribution function with a standard deviation of the particle diameters greater than 1 per cent of the mean diameter. In view of this result, it was concluded that the justification for believing that bushy stunt virus protein particles are strictly homogeneous with respect to size, shape, and density and that they, therefore, may be molecules is as good as for any protein.

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SOME PROPERTIES OF A POLYPHENOLOXIDASE PRESENT IN CELL-FREE KIDNEY EXTRACTS

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Recently Schroeder and Adams (1) have reported that tyrosinase definitely depresses the blood pressure in animals with experimental hypertension. Grollman, Williams, and Harrison (2) as well as Page and his coworkers (3) had previously obtained extracts of kidney which lowered the blood pressure of hypertensive animals. We have prepared extracts as described by these authors and have designed experiments to test for oxidative activity of such extracts.

EXPERIMENTAL

Preparation of Extracts—Extracts were originally prepared by the method of Grollman, Williams, and Harrison (2). Fresh pig kidney was taken from the animal and placed in dry ice in order to be quickly and completely frozen. This was finely ground into 0.25 N hydrochloric acid (2 liters per kilo of fresh tissue) and after standing for 12 hours was filtered through cloth. The extract was then brought to pH 3.9 to 4.1 with 5 N sodium hydroxide, the precipitate filtered off, and the filtrate acidified to pH 2.0 to 3.0. 1.5 volumes of saturated ammonium sulfate were then added, the mixture allowed to stand for several hours, and the precipitate collected on a large Buchner funnel and thoroughly dried with acetone. The precipitate was dissolved in water and dialyzed for 4 hours. The solution was then diluted with water so that 1 cc. represented 60 to 80 gm. of fresh kidney and passed through a Seitz filter.

The method of Page and coworkers (3) was also used to obtain this material. Here the dialyzed solution was made up so that 1 cc. was equivalent to 25 to 50 gm. of fresh tissue.

On the basis of the fact that the material is soluble in aqueous acetone solution and can be precipitated by alcohol or acetone we have prepared extracts by the following method. 1 kilo of ground pig kidneys (obtained as soon as possible after removal from the animals) is added to 1900 cc. of 57 per cent acetone (by volume) and the mixture is stirred well and allowed

to stand overnight. It is then filtered through cloth and 940 cc. of 40 per cent acetone are added to the residue. This mixture is stirred for ½ hour and then filtered through cloth. The combined filtrates are concentrated to a volume of about 1100 cc. under diminished pressure at a temperature not exceeding 50°. The solution is filtered through Carl Schleicher and Schüll No. 1117½ filter paper. To each liter of filtrate 532 gm. of ammonium sulfate are added with constant stirring. The mixture is allowed to stand for a few hours and then filtered through Whatman No. 5 filter paper on a Buchner funnel. The residue is dissolved in water, centrifuged, and then dialyzed in cellophane tubing against distilled water for 3 hours. The water is changed frequently during the 1st hour and then once each half hour.

The solution thus obtained is centrifuged and then passed through a Seitz filter. The solution can be further purified by adding slowly, with stirring, 8 volumes of alcohol to the extract. The mixture is centrifuged and the supernatant fluid discarded. The precipitate is washed several times with water. A considerable portion of the precipitate will remain undissolved but this will contain very little of the material which has enzymatic activity.

A similar procedure may be carried out with acetone. In this case we have used 10 volumes of acetone instead of the 8 volumes of alcohol.

In these methods of extraction the final solution was made up so that 1 cc. would represent 10 to 20 gm. of fresh pig kidney.

Enzymatic Activity—The system recommended by Adams and Nelson (4) for the determination of tyrosinase activity was used. A sodium phosphate-acetic acid buffer (pH 7.0 to 7.2 was arbitrarily chosen), gelatin, enzyme, and substrate (an aqueous solution containing 5 mg. of hydroquinone and 0.1 mg. of catechol per cc.) were placed in a Klett-Summerson (5) colorimeter tube. The rate of oxidation was followed by measuring the development of red color. A green filter, No. 54, was used. In order to ascertain that the development of red color was definitely an oxidative process, the oxygen uptake was measured manometrically[1] in a Warburg apparatus.

Chemical and Physical Properties—In order to study the effect of pH on the rate of reaction, determinations were made colorimetrically at pH values ranging from 7.0 to 8.6. As the pH increases, the rate of reaction is accelerated and as noted also in the manometric determination (pH 7.2) there is a lag of about 10 minutes in the onset of oxidation at the lower

values. The data presented in Fig. 1 were obtained with an extract prepared by the method of Grollman, Williams, and Harrison.

The substance responsible for the oxidation is partially inactivated by heating in a water bath for a period of 1 hour at a temperature of 80–100°. It is precipitated by acetone or alcohol and may be redissolved in water with only slight loss of activity. The enzyme is capable of oxidizing catechol or hydroquinone but is ineffective toward tyrosine, dihydroxy-
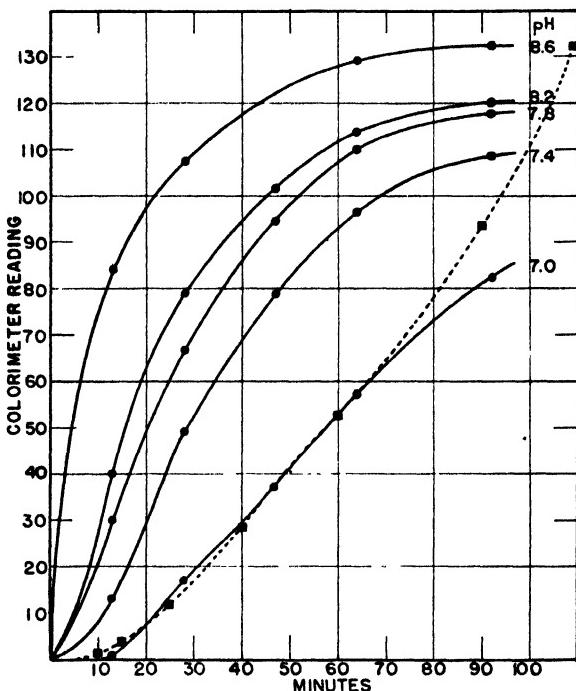
FIG. 1. Curves of the rate of enzymatic activity at various pH levels. The dotted curve represents oxygen uptake and the ordinate in this instance represents c.mm. Na_2HPO_4-citric acid buffers were used for pH 7.0, 7.4, and 7.8. NaOH-boric acid buffers were used for pH 8.2 and 8.6. An aqueous solution containing 5 mg. of hydroquinone and 0.1 mg. of catechol per cc. was used as the substrate. The oxygen uptake of the control in the manometric determination was very slight. Corrections were made in each instance for autoxidation.

phenylalanine (dopa), and p-cresol[1] when these substances are substituted individually for the hydroquinone-catechol solution in the system described under "Enzymatic activity." In view of the inability of the enzyme to oxidize p-cresol and tyrosine we are led to believe that the substance is not tyrosinase. However, it is capable of oxidizing hydroquinone very readily in the absence of catechol. On this basis it seems reasonable to believe that the substance is an oxidase similar to a laccase (6).

In so far as the authors know, this is the first instance in which the presence of such an enzyme has been demonstrated in a cell-free extract of animal tissue.

All of the extracts were given in graded doses to a group of mature rabbits with chronic hypertension produced by partial obstruction of the abdominal aorta proximal to the points of origin of the renal arteries. Blood pressure was taken from the auricular artery by palpation (7). A delayed drop of the blood pressure of more than 30 mg. of mercury maintained for 48 hours or more was considered significant. We were unable definitely to correlate enzyme activity and blood pressure-lowering activity in these extracts. However, processes for the purification and standardization of the enzyme are being worked out and a further study of the blood pressure-lowering activity will be made.

SUMMARY

A cell-free kidney extract has been prepared which is capable of oxidizing certain polyphenols; namely, catechol and hydroquinone. It is ineffective in oxidizing either tyrosine, dihydroxyphenylalanine (dopa), or p-cresol. The substance is partially inactivated by heat, is precipitated by either alcohol or acetone without unreasonable loss of activity, and is not dialyzable. From the data it appears that the substance is similar to a laccase.

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THE LIPIDS OF THE DUODENAL MUCOSA OF SWINE DURING THE ABSORPTION OF FAT

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Most modern studies of the fat content of the intestinal mucosa during fat absorption have been concerned with the changes in the quantity and quality of the phospholipids. Sinclair's classical paper (1) in which he reported no change in amount but a pronounced change in composition of mucosa phospholipid during fat absorption set the style. Investigations of the other lipids of the mucosa during absorption are few and incomplete. Sperry (2) found that the total lipid of the fasting mucosa was 7.8 per cent and the unsaponifiable matter 1.47 per cent. Lovern and Morton (3) in an investigation of the esterification of fatty acids with vitamin A and cholesterol during absorption of fat by fishes reported analyses of the oil of fish intestines but made no effort to determine the content of the common lipids in the intestine or mucosa. They did report, however, that intestinal oils "extracted only a few minutes after the death of the fish frequently contained up to 30 per cent free fatty acids." Recently Barnes, Miller, and Burr (4) investigated the rate of the incorporation of conjugated double bond fatty acids prepared from corn oil into the acetone-soluble and insoluble lipid fractions of the intestinal mucosa of fats during their absorption. They found that the acetone-soluble lipids increased from 20.6 per cent in the mucosa of the fasting animal to 37.6 per cent 1 hour after the oil was placed in the duodenum.

Holmgren (5) in a combined chemical and histological study of rats found a diurnal variation in the amount of fat in the entire intestine with the minimum value at 2 p.m. and the maximum at 2 a.m. Unfortunately, figures were given only for gm. of fat in the total intestine per kilo of body weight. The average fat content of the intestine after a 48 hour fast was found to be 1.34 per cent of the fresh tissue. Rats fasted 48 hours and then allowed to eat *ad libitum* for 3 hours before death showed an intestinal fat content of 2.26 per cent when killed at 2 p.m. and 3.72 per cent when killed at 10 p.m. Assuming the intestinal contents are 18 per cent solid

matter (6), these fasting and postprandial values are 7.40, 12.55, and 20.67 per cent respectively.

There have been numerous histological studies of this subject. The better known modern investigations are those of Mottram, Cramer, and Drew (7), Cramer and Ludford (8), and the more recent work of Jeker (9), of Wotton and Zwemer (10), and of Holmgren (5). All the histological studies show that the columnar cells of the intestinal epithelium, though apparently free of fat in the fasting condition, become engorged with it during the active absorption of fat. From the drawings and photomicrographs presented by these authors it would appear that fat fills the cells to about 50 per cent of their volume during the most active period of absorption. Such a tremendous increase in the fat content of a tissue should be readily detected chemically and the present investigation was undertaken with that objective. It was hoped also that the knowledge of all the lipid changes during absorption might throw some light on the still obscure mechanism of that phenomenon.

Methods

The experimental animals used were pure bred Duroc pigs of both sexes, about 7 months old and weighing approximately 225 pounds. They had been on experiment to determine the relative values of different protein supplements in their nutrition. All had been on the same diet for several weeks before these experiments.

Four groups of five animals each were used. After fasting 48 hours one group was slaughtered in the fasting state, one group 3 hours after the ingestion of 200 gm. of cottonseed oil in about 1 pound of a standard feed, another group 3 hours after ingestion of 300 gm. of cottonseed oil in 1 pound of feed, and the fourth group 5 hours after the latter meal. The animals were killed by exsanguination, and the mucosa washed and scraped from the first meter of intestine and immediately frozen in carbon dioxide.

Extraction—A 4 gm. sample was weighed in a 6 inch test-tube and 3:1 alcohol-ether mixture added to within an inch of the top. The mucosa was stirred with a glass rod, stoppered, and allowed to stand until ready for analysis. The tube was then heated to boiling, centrifuged, and the supernatant solution decanted into a 250 cc. Erlenmeyer flask. This extraction was repeated twice and the material then extracted once with ether alone. The residue was ground in a mortar, without sand, and the fine powder extracted several times with boiling alcohol-ether mixture and finally twice with ether. The combined extracts were evaporated to dryness by applying a vacuum to the Erlenmeyer flask and rotating. Frothing toward the end was greatly reduced by adding 2 drops of caprylic alcohol. A little

absolute alcohol also aided in washing out the last traces of water. The dried material in the flask was extracted with petroleum ether, centrifuged to clear the extract of suspended material, and made up to 100 cc. in a volumetric flask.

Phospholipid—A 3 cc. aliquot of the petroleum ether solution was analyzed for phosphorus according to King's modification (11) of the Fiske-Subbarow procedure. The amount of phosphorus was multiplied by the factor 26 to obtain the amount of phospholipid.

Free Fatty Acids—25 cc. of the petroleum ether solution were evaporated to about 0.5 cc. in a 50 cc. conical shaped centrifuge tube. The phospholipid was separated by adding 2 drops of a saturated alcoholic solution of strontium chloride and 15 cc. of acetone. After standing at least an hour the tubes were centrifuged and the supernatant solution and the acetone washings of the precipitate made up to 25 cc. A 10 cc. aliquot of this solution was transferred to a 22 × 75 mm. weighing bottle, evaporated to dryness, and the free fatty acids titrated according to the method previously described by the author (12) except that 0.02 N sodium hydroxide was used and the titration was carried out at the boiling temperature without the second titration. Fatty acids were calculated as oleic.

Triglycerides—A second 10 cc. aliquot of the acetone solution was evaporated to dryness in a 50 cc. Erlenmeyer flask, and the lipids dissolved in 5 cc. of 95 per cent alcohol and saponified with 0.1 cc. of saturated potassium hydroxide. The solution was heated on the water bath for 1 hour and the alcohol removed by a current of air. The soaps were decomposed with 1 cc. of dilute sulfuric acid and the fatty acids extracted with petroleum ether. The combined petroleum ether extracts were centrifuged to remove any small suspended drops of sulfuric acid, transferred to the titration bottles, evaporated to dryness, and the fatty acids titrated, as were the free fatty acids. The total fatty acids minus the free and cholesterol fatty acids were considered as triglyceride fatty acids.

Total Cholesterol—Total cholesterol was determined on a 3 cc. aliquot of the acetone solution according to the colorimetric method of Bloor (13). It was found that the brown color so often troublesome in this determination could be eliminated by using the phospholipid-free extract.

Ester Cholesterol—The phospholipid was precipitated from a 10 cc. aliquot of the petroleum ether solution of the total fat and the acetone washings evaporated to dryness in a 50 cc. beaker. The free cholesterol was precipitated by dissolving the fat in a little petroleum ether and adding 3 cc. of a 0.2 per cent solution of digitonin in 50 per cent alcohol and slowly evaporated to dryness. The cholesterol ester was extracted with chloroform and determined colorimetrically.

DISCUSSION

The results of the analyses are given in Table I. There was no increase in the phospholipids or the cholesterol of the absorbing mucosa. The free fatty acids were approximately doubled in amount 5 hours after ingestion, but it is questionable whether there were any triglycerides in the mucosa at any time. It is possible that a small amount made its appearance during the 3rd hour, but since this was determined by difference such

TABLE I

Lipids of Duodenal Mucosa of Swine during Fat Absorption

Pig No.	Oil fed	Time after meals	Phospho-lipid	Cholesterol		Free fatty acids	Triglyc-eride acids	Total fat
				Total	Ester			
	gm.	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	None	Fasting	10.1	1.66	0.49	2.98	−0.28	15.2
2	"	"	10.5	1.61	0.46	2.49	−0.31	15.1
3	"	"	10.0	1.70	0.47	2.94	0.36	15.1
4	"	"	10.5	1.59	0.41	1.89	0.56	14.9
5	"	"	10.0	1.86	0.33	2.66	0.56	15.4
Mean	"	"	10.2	1.68	0.43	2.59	0.17	15.1
6	200	3		1.77	0 56	3.37	−0.41	
7	200	3		1.73	0 62			
8	200	3		1.69	0 39	2.40	0.10	
9	200	3		1.79	0.38	3.39	0.29	
10	200	3		1.33	0.36	4.60	1.21	
Mean	200	3		1.66	0.46	3.44	0.30	
11	300	3	11.5	1.59	0.32	3.98	0.00	17.4
12	300	3	9.4	1.64	0.34	3 47	3.84	18.7
13	300	3	9.9	1.69	0 35	4.73	1.34	18.0
14	300	3	10.1	1 36	0.41	4.83	1.01	17.7
15	300	3	10.4	1 49	0.47	4.51	1.52	18.4
Mean	300	3	10.3	1.55	0.38	4.30	1.54	18.0
16	300	5	10.1	1.64	0.51	6.28	3.42	21.9
17	300	5	10.3	1.26	0.13	4.18	−0 75	15.9
18	300	5	11.2	1.46		6.85	−1.03	
19	300	5	10.8	1.60	0.28	4.23	1.66	18.6
20	300	5	10.8	1.67	0.41	4.80	0.00	17.7
Mean	300	5	10.6	1.53	0.33	5.26	0.66	18.5

small amounts must be questioned and, though present, could not be of much significance.

It would thus appear that the only significant change in the lipids of the absorbing mucosa is a maximum increase of about 100 per cent in the free fatty acids. Including a possible increase in triglycerides, this represents an increase of 3.0 or 3.5 per cent in the total lipid content of the dry mucosa or 0.6 per cent (6) of the fresh tissue. This slight change in fat content could by no means account for the very great increase in stainable

fat observed in the histological studies mentioned above. However, the chemical analyses differ from the histological evidence not so much in that they fail to show the increase in fat during absorption but in that they prove the presence of considerable fat in the fasting mucosa not observed histologically. The apparently great increase in fat observed histologically does not, then, represent an increase in the fat content of the mucosa cells but an increase in the amount of stainable fat.

It is generally accepted as fact (14, 15) that fats that are a living part of the protoplasmic structure are not readily stainable by histological procedure. From this fact and the data just presented it would appear that in the resting mucosa cells the lipids are a definite part of the protoplasm and are not stainable, but that during absorption there is a change in their physicochemical state and they then exist as individual compounds. In that state they are stainable.

Macheboeuf (16) has presented evidence that "living" fats are combined with protein and are not only not stainable for histological examination but are also insoluble in ether until after treatment with alcohol. This difference in solubility offers a test of the hypothesis presented above. If the hypothesis is correct, extraction of non-absorbing and of absorbing mucosa with ether should show very little fat in the former but considerable quantities in the latter. That is, it should give results corresponding to those of histological examination. Support comes from the older literature and from Holmgren (5). Noll in 1911 (17) dried intestinal mucosa and then extracted it with petroleum ether. In the fasting tissue he found about 5 per cent total fat, while in the absorbing cells he found from 10 to 30 per cent. Ferrata and Moruzzi in 1907 (18) using the same procedure found that the total fat, on the fresh basis, increased from 1.06 to 2.40 per cent. Recalculated on the dry basis these figures would be approximately 6.0 and 13.3 per cent. These figures compare well with those of Holmgren (5) who found, by ether extraction, 7.40 per cent of fat in the fasting mucosa and 12.55 to 20.67 per cent in the absorbing tissue, depending on the hour.

Thus, the figures obtained by the ether extraction of the dried absorbing mucosa are of the same degree as those obtained by alcohol-ether extraction, but the ether extraction of the fasting mucosa gives values as little as one-eighth as large as does the alcohol-ether procedure.

In a very recent report Barnes, Miller, and Burr found a total of 31.2 per cent of fat in the fasting mucosa of rats and 49.6 per cent 1 hour after the methyl esters of conjugated double bond fatty acids prepared from corn oil were placed in the duodenum. These values are in sharp contrast to those presented in the present paper. One possible explanation is that of species difference. Other possibilities are the differences due to method of administration and time. Barnes and his coworkers placed the fat in

the duodenum and got a maximum effect in 1 hour and a return almost to the fasting level in 2 hours.   In the present work the fat was ingested and the tissue removed 3 and 5 hours later.   These differences, however, do not affect the major thesis.   Barnes and his coworkers found that almost one-third of the fasting mucosa is fat as compared to the ether extraction values of 5 to 7 per cent and the histological evidence of almost none. These authors also found that about half of the dry weight of the absorbing mucosa is fat, a figure which approximates the results of ether extraction and the evidence of histological procedure.

SUMMARY

1. There is no change in the phospholipid or cholesterol content of the duodenal mucosa of swine during absorption.

2. There are no triglycerides in the fasting duodenal mucosa of swine and only small amounts, if any, during absorption.

3. About 2.5 per cent of the dried weight of fasting mucosa is free fatty acid and this amount is approximately doubled 5 hours after the ingestion of oil.

4. A theory is presented to explain the apparent differences presented by the histological and chemical studies of the absorbing mucosa.

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THE RELATION OF ASPARTIC ACID AND GLUCOSAMINE TO GROWTH*

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In recent years evidence has been presented for the dietary indispensability of phenylalanine (1), threonine (2), leucine (3), isoleucine (3), methionine (4), and valine (5). Previously, the essential nature of lysine (6), tryptophane (6), and histidine (7, 8) had been established. On the other hand, tyrosine (1), norleucine (3), glycine (9), serine (9), cystine (4), and alanine (10) are dispensable components of the food of the mammal inasmuch as they can be synthesized *in vivo* out of materials ordinarily available. Furthermore, the numerous feeding experiments in this laboratory, in which excellent growth has been obtained *without* the inclusion of hydroxyglutamic acid and citrulline in the rations, demonstrate that these amino acids also are non-essential. Thus, seventeen components of proteins have been definitely classified with respect to their growth significance.

In the present paper the rôle of aspartic acid and glucosamine is described. Little information is available concerning the nutritive importance of either of these compounds. Many years ago, Hopkins (11) expressed the opinion that aspartic acid is dispensable. A similar conclusion was reached by St. Julian and Rose (12) as the result of experiments in which the nitrogen of the diet was furnished in the form of hydrolyzed casein from which the dibasic amino acids had been removed according to the procedure of Kingston and Schryver (13). It was recognized, however, that traces of aspartic acid might have remained in the hydrolyzed protein, since no delicate test is available for the detection of this amino acid. Abderhalden (14) believes that aspartic acid is an essential dietary constituent, but the experimental data upon which this conclusion is based are not impressive.

With respect to the physiological significance of glucosamine, even less is known. Rimington (15) and others have found this compound to be present in a number of proteins, but apparently no studies of its nutritive importance have previously been made. The diets used in our earlier experiments always carried 1 per cent of glucosamine hydrochloride in order to exclude the possibility of a deficiency from this source.

TABLE I

Composition of Amino Acid Mixture

| | Mixture XII-c | |
	Active amino acids	As used
	gm.	*gm.*
Glycine	3.00	3.00
Alanine.................	1.90	3.80*
Valine........	8.00	16.00*
Leucine...	9.00	9.00
Isoleucine	4.00	8.00*
Norleucine...	1.25	2.50*
Proline.....	8.00	8.00
Hydroxyproline..	2.00	2.00
Phenylalanine	3.90	7.80*
Glutamic acid	22.00	22.00
Aspartic "	0	0
Serine...	1.50	3.00*
Tyrosine.	6.50	6.50
Cystine.	1.25	1.25
Histidine.	2.52	
" monohydrochloride monohydrate		3 40
Arginine	5.25	
" monohydrochloride........		6.35
Lysine.	7.70	
" dihydrochloride............·...		11.55
Tryptophane	2.25	2.25
Methionine..	1.75	3.50*
Sodium bicarbonate.		12.86
	91.77	132.76†

* Racemic acids.

† 1.447 gm. of mixture are equivalent to 1.0 gm. of natural amino acids.

EXPERIMENTAL

Young white rats were used as the experimental animals. As in the other investigations of this series, a mixture of highly purified amino acids served as the source of nitrogen in formulating the diets. The composition of this mixture (Mixture XII-c) is shown in Table I. As will be observed, it was devoid of threonine and aspartic acid. These amino acids, and gluco-

samine in the form of the hydrochloride, were incorporated in the diets separately. All of the nitrogenous components of the food were shown to be analytically pure. In order to insure the absence of traces of aspartic acid, which might have been present as a contaminant of the glutamic acid, the latter was recrystallized three times after it yielded correct analytical values.

The make-up of the diets is presented in Table II. Each diet supplied 18 per cent of natural amino acids (including glucosamine when present), and was consumed by the animals *ad libitum*. Diet 1 contained both glucosamine hydrochloride and aspartic acid, and served as the positive con-

TABLE II

*Composition of Diets**

	Diet 1	Diet 2	Diet 3
	gm	*gm.*	*gm.*
Amino acid Mixture XII-c	22.8	24.0	23.8
Threonine (*d*)†	0.7	0.7	0.7
Dextrin	22 4	22 6	22.1
Sucrose	15.0	15.0	15.0
Salt mixture‡	4.0	4.0	4 0
Agar. . . .	2 0	2.0	2.0
Lard	26 0	26 0	26 0
Cod liver oil	5 0	5.0	5.0
Aspartic acid (*l*)	0 7	0.7	0
Glucosamine hydrochloride (*d*)	1 0	0	1.0
Sodium bicarbonate	0.4	0	0.4
	100.0	100 0	100.0

 * Each diet contained 18 per cent of natural amino acids, including glucosamine The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from these sources amounted to approximately 4 mg.

  † Natural $d(-)$-threonine (*cf.* Meyer and Rose (16)).

  ‡ Osborne and Mendel (17).

trol ration. Diet 2 contained aspartic acid but was devoid of glucosamine, and Diet 3 was free of aspartic acid but contained 1 per cent of glucosamine hydrochloride. To Diets 1 and 3 was added a sufficient quantity of sodium bicarbonate to neutralize the hydrochloric acid in the glucosamine hydrochloride. The vitamin B factors were furnished to each animal in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily nitrogen intake from these sources amounted to approximately 4 mg., and was the only nitrogen of unknown kind in the rations.

The results of the experiments are summarized in Table III. All of the

subjects grew rather more rapidly than is usually observed upon "synthetic" diets of this nature. Possibly the particular samples of milk concentrate and tikitiki used in these tests were above the average in their content of the vitamin B factors. In any event, the data in Table III demonstrate that the ability of animals to gain in weight is not inhibited by depriving them of either glucosamine or aspartic acid. Indeed, the variations within each litter are not greater than are encountered frequently among animals receiving the same diet.

TABLE III

Total Changes in Body Weight and Total Food Intakes of Experimental Animals
The experiments covered 28 days each.

Litter No.	Rat No. and sex	Total increase in weight	Total food intake	Supplement
		gm.	gm.	
1	2464 ♀	70	180	No glucosamine
	2465 ♂	69	195	" "
	2466 ♂	74	199*	" "
	2467 ♀	68	199*	" "
	2468 ♀	64	177	" "
	2469 ♂	61	170	1% d-glucosamine hydrochloride
	2470 ♀	69	195	1% " "
	2471 ♀	65	171	1% " "
	2472 ♀	56	158	1% " "
2	2493 ♂	62	188*	0.7% l-aspartic acid
	2494 ♂	49	198*	0.7% " "
	2495 ♀	57	169	0.7% " "
	2496 ♀	54	200*	0 7% " "
	2497 ♂	60	165	No aspartic acid
	2498 ♂	51	165	" " "
	2499 ♀	61	149	" " "
	2500 ♀	56	187*	" " "
	2501 ♀	56	175*	" " "

* Animal scattered food; recorded intake is probably too high.

A somewhat clearer picture of the growth *progress* of the members of Litter 2 is shown by the curves of Chart I. Obviously, the subjects which received aspartic acid did not manifest a more rapid *rate* of gain than did their litter mates which were denied access to this amino acid. This is true also of the animals used in the glucosamine experiment (Litter 1), the growth curves of which are omitted in order to conserve space. Evidently, both *glucosamine and aspartic acid are dispensable components of the food.* This conclusion has been confirmed repeatedly since the above experiments were completed, by the use of simplified diets containing neither aspartic acid nor glucosamine.
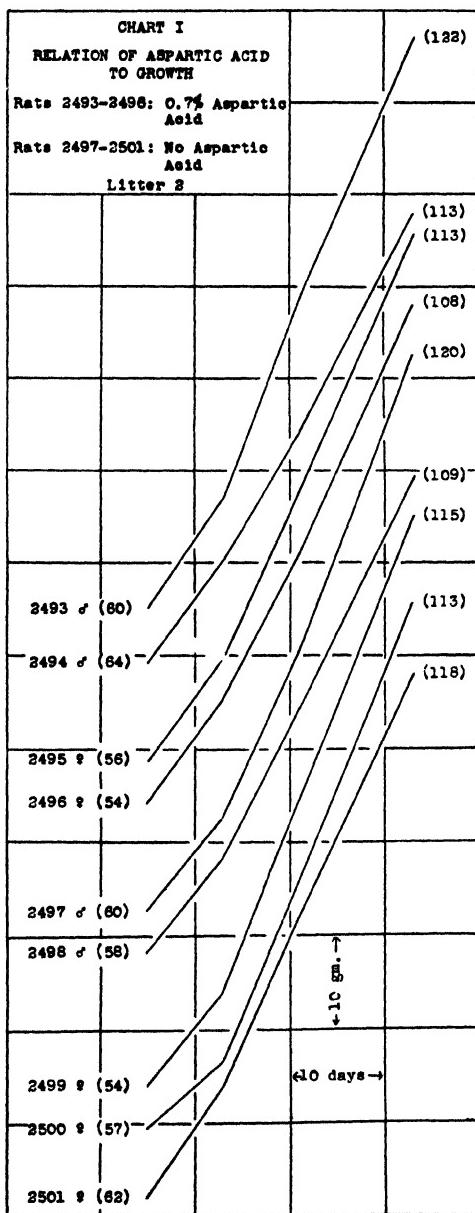
FIG. 1. The numbers in parentheses denote the initial and final weights of the rats.

SUMMARY

By the use of diets devoid of proteins, but containing mixtures of highly purified amino acids, *aspartic acid and glucosamine have been shown to be dispensable components of the food.* Rats deprived of either of these compounds increase in body weight just as rapidly as do litter mate controls receiving a like ration supplemented with the compound in question.

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AROMATIC SULFONIC ACIDS AS REAGENTS FOR AMINO ACIDS

THE PREPARATION OF *l*-SERINE, *l*-ALANINE, *l*-PHENYLALANINE, AND *l*-LEUCINE FROM PROTEIN HYDROLYSATES

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A number of sulfonic acids of the benzene and naphthalene series have recently been recommended as reagents for amino acids (1). The experiments described in the present communication extend the earlier work to sulfonic acids derived from diphenylamine, anthraquinone, and azobenzene and also illustrate how a number of these reagents may be employed to advantage for the preparation of amino acids from protein hydrolysates.

The structure of a number of the sulfonic acids investigated, together with the solubility products of their amino acid salts, is given in Table I. In general the solubilities listed in Table I are lower than those of the amino acid salts of the benzene- and naphthalenesulfonic acids previously reported. Several of the reagents warrant special mention. The *l*-isoleucine salt of 4-nitro-4'-methyldiphenylamine-2-sulfonic acid (Reagent 7) is considerably less soluble than are the corresponding *l*-leucine and *l*-phenylalanine salts. Its application to the isolation and determination of isoleucine, therefore, may merit investigation. Five of the azobenzenesulfonic acids (Reagents 1, 2, 4, 5, and 6) and the three anthraquinonesulfonic acids (Reagents 10, 11, and 12) all form sparingly soluble salts with most of the amino acids tested. Remarkably low in solubility are the arginine and histidine salts of 3-carboxy-4-hydroxyazobenzene-4'-sulfonic acid (Reagent 4) and 2-hydroxy-5-methylazobenzene-3'-sulfonic acid (Reagent 6) and the glycine, arginine, histidine, and lysine salts of 5-nitroanthraquinone-1-sulfonic acid. Among this group of reagents *p*-hydroxyazobenzene-*p'*-sulfonic acid (Reagent 2) is also noteworthy because it is capable of precipitating *l*-serine. This reagent has been utilized for the isolation of *l*-serine from silk fibroin (2). It is interesting that the isomeric *p*-hydroxyazobenzene-*m'*-sulfonic acid (Reagent 3) behaves entirely differently. In this case the *l*-phenylalanine salt is much less soluble than are any of the

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other amino acid salts. The utility of this substance is impaired, however, by the insolubility of the free sulfonic acid itself. Finally, it should be noted that many of the aforementioned azo compounds, even in strongly acidic solutions, form salts with a number of organic bases such as

TABLE I—*Solubility Products of Amin*

The solubilities were determined at 0°. It should be emphasized that all the ; in the experimental section. Where no value is given, no precipitation was obtained

Amino acid	(1) Azobenzene-p-*	(2) p-Hydroxy-azobenzene-p'-†	(3) p-Hydroxy-azobenzene-m'-‡	(4) 3-Carboxy-4-hydroxyazo-benzene-4'-	(5) 3-Carboxy-4-hydroxyazo-benzene-3'-
l-Alanine	4×10^{-4}	2×10^{-3}			
Ammonia	4×10^{-2}	4×10^{-5}	3×10^{-4}	3×10^{-3}	3×10^{-4}
l-Arginine**	2×10^{-8}	2×10^{-8}		1×10^{-9}	5×10^{-9}
l-Aspartic acid					
l-Cysteine	2×10^{-5}	8×10^{-6}		4×10^{-6}	1×10^{-5}
l-Cystine**	1×10^{-6}	8×10^{-8}		2×10^{-9}	3×10^{-7}
l-Glutamic acid		4×10^{-3}			
Glycine	2×10^{-4}	1×10^{-3}		3×10^{-2}	5×10^{-4}
l-Histidine**	3×10^{-8}	2×10^{-9}		7×10^{-10}	2×10^{-9}
l-Hydroxyproline					2×10^{-3}
l-Isoleucine	4×10^{-4}			2×10^{-4}	6×10^{-4}
l-Leucine	8×10^{-5}	3×10^{-4}		1×10^{-4}	1×10^{-4}
l-Lysine**.	1×10^{-7}	3×10^{-8}		1×10^{-8}	2×10^{-8}
dl-Methionine	6×10^{-5}	2×10^{-4}		4×10^{-5}	1×10^{-5}
l-Phenylalanine	1×10^{-5}	6×10^{-6}	4×10^{-5}	1×10^{-5}	4×10^{-5}
l-Proline		3×10^{-4}			
l-Serine		2×10^{-4}			
dl-Threonine					
l-Tyrosine		1×10^{-4}		8×10^{-6}	2×10^{-5}
l-Valine	1×10^{-4}			1×10^{-3}	

* 0.3 cc. of methyl cellosolve added to each sample to dissolve the free sulfonic acid
† Sulfonic acid dissolved in 40 per cent methyl cellosolve.
‡ Sulfonic acid dissolved in 50 per cent methyl cellosolve, and 1 cc. of water and
§ 0.1 cc. of methyl cellosolve added to each sample to dissolve the free sulfonic
‖ 0.2 cc. of methyl cellosolve and 1 cc. of water added to each sample to dissolve
¶ Sulfonic acid dissolved in n HCl; solubilities determined in n HCl.
** The values given for these amino acids represent the solubility products of the

piperidine, aniline, pyridine, hydroxylamine, β-aminophenol, ethanolamine, creatine, and urea.

Picrylsulfonic acid (Reagent 13), like picric acid, forms sparingly soluble salts with ammonia, the three basic amino acids, and with glycine and proline. This reagent, which in contrast to picric acid may be employed

in strongly acid solution, gives promise of being useful for the determination of proline.

The following sulfonic acids have also been investigated: 2,6-dinitro-2'-methyldiphenylamine-4-, 2,6-dinitro-3'-methyldiphenylamine-4-, 4,6-

cid Salts of Aromatic Sulfonic Acids

ubility products are approximate values only, estimated in the manner described t concentrations corresponding to a solubility product of 4×10^{-2}.

ubility products of sulfonic acid salts

(6) Hydroxy-methylazo-benzene-3'-§	(7) 4-Nitro-4'-methyldiph-enylamine-2-	(8) 2-Nitro-4'-methyldiph-enylamine-4-	(9) 4-Nitro-diphenyl-amine-2-	(10) Anthraqui-none-α-	(11) Anthraqui-none-β-	(12) 5-Nitro-anthraqui-none-1-‖	(13) Picryl-¶
				3×10^{-3}	2×10^{-3}	6×10^{-4}	
$\times 10^{-3}$	4×10^{-2}	2×10^{-3}	4×10^{-4}	6×10^{-5}	3×10^{-3}	1×10^{-4}	4×10^{-4}
$\times 10^{-10}$	Oil	4×10^{-9}	3×10^{-7}	2×10^{-8}	3×10^{-9}	2×10^{-9}	8×10^{-6}
						2×10^{-3}	
$\times 10^{-6}$		2×10^{-3}		6×10^{-4}		4×10^{-5}	
$> 10^{-8}$	Oil		Oil	8×10^{-7}	3×10^{-8}	8×10^{-9}	
$\times 10^{-2}$							
$\times 10^{-5}$			5×10^{-4}	2×10^{-4}	1×10^{-2}	4×10^{-6}	3×10^{-3}
$\times 10^{-9}$	2×10^{-8}	2×10^{-8}	3×10^{-7}	3×10^{-9}	3×10^{-8}	8×10^{-9}	2×10^{-5}
$\times 10^{-2}$						3×10^{-3}	
$\times 10^{-2}$	8×10^{-6}	1×10^{-4}		3×10^{-4}	2×10^{-4}	3×10^{-3}	
$\times 10^{-6}$	2×10^{-4}	8×10^{-5}	5×10^{-4}	5×10^{-4}	1×10^{-4}	2×10^{-5}	
$\times 10^{-7}$	Oil	4×10^{-7}	6×10^{-9}	6×10^{-8}	1×10^{-6}	3×10^{-9}	3×10^{-4}
$\times 10^{-6}$	7×10^{-6}	4×10^{-5}		1×10^{-4}	3×10^{-3}	2×10^{-4}	
$\times 10^{-6}$	5×10^{-4}	2×10^{-4}	3×10^{-5}	1×10^{-3}	3×10^{-5}	6×10^{-5}	
$\times 10^{-1}$	3×10^{-5}					3×10^{-4}	5×10^{-4}
				2×10^{-3}		4×10^{-3}	
						6×10^{-4}	
					2×10^{-3}		
$> 10^{-3}$	1×10^{-5}	8×10^{-5}		1×10^{-3}	4×10^{-3}	1×10^{-3}	

cc of methyl cellosolve added to each sample to dissolve the reagent.
d.
∶ free sulfonic acid.

nary salts (see foot-note to Table I (1)).

dinitrodiphenylamine-2-, 4,6-dinitro-2'-methyldiphenylamine-2-, 2-nitro-3'-methyldiphenylamine-4-, 2-nitro-4'-ethoxydiphenylamine-4-, 4-nitrodiphenylamine-2-, 2-benzylamino-5-nitrobenzene-, and 4-nitro-4'-methyldiphenylamine-2-. Their amino acid salts possess, in general, solubilities similar to those of the reagents given in Table I.

The utilization of sulfonic acids for the preparation of amino acids may be exemplified by the procedures for the isolation of *l*-phenylalanine and *l*-leucine from a hydrolysate of hemoglobin, and of *l*-serine and *l*-alanine from a hydrolysate of silk fibroin.

In the course of the preparation of lysine (3) and histidine from hemoglobin an insoluble amino acid fraction is obtained. From this by-product we have prepared, per kilo of commercial hemoglobin, about 48 gm. of *l*-leucine (free of methionine, isoleucine, and valine) with the aid of 2-bromotoluene-5-sulfonic acid, and 20 gm. of *l*-phenylalanine with the aid of 2,5-dibromobenzenesulfonic acid. If the preparation of *l*-phenylalanine is not desired, *l*-leucine may be obtained more economically by purification of commercial products with the aid of naphthalene-β-sulfonic acid (4).

The methods hitherto employed for the preparation of *l*-phenylalanine are discussed by Baptist and Robson (5). These authors proposed a procedure for the isolation of this amino acid utilizing both its copper salt and its picrolonate, and applied the procedure to hydrolysates of zein and casein. The use of dibromobenzenesulfonic acid would appear to be simpler and more economical with respect to time and material.

The natural *l*-serine has heretofore not been readily available. The classical method for its preparation is that of Fischer and Jacobs (6) who employed alkaloids to resolve *p*-nitrobenzoyl-*dl*-serine. The preparation of *l*-serine from hydrolysates of silk fibroin with the aid of hydroxyazobenzenesulfonic acid was recently reported (2). The detailed procedure is described in the experimental section of this communication. In addition to the 95 gm. of *l*-serine obtained from each kilo of fibroin, 240 gm. of *l*-alanine are isolated as a by-product.

EXPERIMENTAL

The solubility products listed in Table I were obtained by a procedure similar to that reported previously (1), with the following modifications: The amino acids were dissolved in N HCl as before, but the sulfonic acids, used as reagents, were dissolved in water. Similarly, in order to determine the solubilities of the amino acid salts formed, each sample was progressively diluted with water instead of N HCl. Finally, whenever a sulfonic acid employed as reagent was found to be sparingly soluble in 0.5 N HCl at 0°, a sufficient quantity of methyl cellosolve was added to each sample to keep the sulfonic acid in solution.

Preparation of l-Leucine and l-Phenylalanine from Hemoglobin—2 kilos of hemoglobin (Eastman, Technical) were hydrolyzed with 6 liters of sulfuric acid (25 per cent by volume), the sulfuric acid removed exactly with barium hydroxide, and the filtrate and washings concentrated *in*

vacuo to 2.4 liters. The insoluble amino acids which separated during the concentration were removed by filtration. The concentrated hydrolysate was stored at 0° overnight, and an additional crop of insoluble material was obtained. The combined yield was about 500 gm. The filtrate served for the isolation of lysine by the method of Rice (3) and subsequently for the isolation of histidine.

For the isolation of *l*-leucine, 1 kilo of the insoluble amino acid mixture was ground to a fine powder, dissolved in 7.5 liters of boiling water, and decolorized with 250 gm. of acid-washed charcoal. To the hot solution 1 liter of concentrated HCl was added, followed by about 1200 cc. of a solution of 2-bromotoluene-5-sulfonic acid, obtained from 850 gm. of the sodium salt over the barium salt[1] (1). The *l*-leucine salt crystallized from the solution at 0°. The filtrate (Solution A) was saved for the isolation of *l*-phenylalanine. The leucine salt was recrystallized from 2.5 liters of water; yield, 925 gm.

To obtain free leucine, the salt was dissolved in 2 liters of hot water, and a solution of 370 gm. of $BaCl_2 \cdot 2H_2O$ in 500 cc. of hot water added. The mixture was stored at 0° overnight and the precipitate of barium 2-bromo-toluene-5-sulfonate saved for recovery of the reagent.[2] The filtrate and washings were concentrated *in vacuo* to about 1.5 liters and neutralized with concentrated NH_4OH. The yield of crude leucine, obtained from the mixture at 0°, was about 200 gm. An additional 30 to 40 gm. may be obtained by further concentration of the mother liquor.

For purification, the crude leucine was dissolved in 2 liters of warm concentrated NH_4OH, and the solution decolorized with acid-washed charcoal and placed in an oil bath at 110–120° in order to boil off the ammonia. Concentration of this solution to about 1 liter yielded 160 gm. of sulfur-free leucine, $[\alpha]_D^{26} = +15.5°$ (5 per cent in 21 per cent HCl). Further concentration of the mother liquor yielded an additional 45 gm. of leucine which was submitted to recrystallization.

For the isolation of *l*-phenylalanine, 300 gm. of 2,5-dibromobenzene-sulfonic acid were dissolved with heating in the aforementioned Solution A. The solution was stored overnight at 0°. The yield of *l*-phenylalanine dibromobenzenesulfonate was 300 to 325 gm. The crude salt was re-crystallized from 2400 cc. of a solution of 1 part of methyl cellosolve and

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[1] The free 2-bromotoluene-5-sulfonic acid is not readily isolated from aqueous solution.

[2] The barium salt was decomposed with aqueous sulfuric acid. The aqueous solution of the free acid thus obtained was usually colored. Addition of $CuCl_2$ (analytical reagent), followed by H_2S, decolorized the solution without introducing ash. If desired, the sodium salt may be obtained from the solution by salting-out with NaCl. In this manner 50 to 60 per cent of the starting reagent may be recovered in pure form.

9 parts of water, and the hot solution decolorized with acid-washed charcoal. Yield, about 225 gm. An additional 50 to 60 gm. of salt were recovered by concentrating the mother liquor *in vacuo* to 600 cc.

To 225 gm. of the recrystallized phenylalanine salt were added 250 cc. of water and 50 cc. of pyridine.[3] The mixture was heated until a clear solution resulted, and 500 cc. of hot absolute alcohol were added. The mixture was kept at 0° and the phenylalanine obtained washed with alcohol and ether; yield, about 55 gm. The filtrate (Solution B) was worked up as described below. The crude phenylalanine was recrystallized from a mixture of 260 cc. of water, 40 cc. of concentrated NH_4OH, and 600 cc. of alcohol in the manner already described for the recrystallization of leucine. Yield, about 35 gm., $[\alpha]_D^{26} = -34.0°$ (2 per cent in water). An additional 10 gm. of phenylalanine were obtained on concentration of the mother liquor.

Solution B, containing phenylalanine dibromobenzenesulfonate and free dibromobenzenesulfonic acid, was concentrated *in vacuo* to 250 cc., warmed, and 175 cc. of concentrated HCl were added. The precipitate obtained by filtration at 0° was stirred vigorously with 375 cc. of water. About 50 gm. of phenylalanine dibromobenzenesulfonate failed to dissolve, and were kept for resubmission to the pyridine treatment. To the aqueous filtrate, from which the 50 gm. of phenylalanine salt had been removed, concentrated HCl was added, and 75 gm. of dibromobenzenesulfonic acid were recovered.

Sodium 2-Bromotoluene-5-sulfonate (7)—Add 500 gm. of *o*-bromotoluene slowly to 500 cc. of concentrated H_2SO_4 plus 500 cc. of fuming H_2SO_4 (25 per cent SO_3). Keep the temperature below 50°; allow the reaction mixture to stand for 20 minutes and pour into 8 liters of ice. The sodium salt is obtained by salting-out with NaCl, and is recrystallized from water.

2,5-Dibromobenzenesulfonic Acid (8)—Heat 500 gm. of *p*-dibromobenzene for 1 hour on the steam bath with 1 liter of fuming sulfuric acid (25 per cent SO_3). Pour the mixture into 6 liters of ice. Heat, filter the hot solution, and cool the filtrate to 0°. The sulfonic acid which separates is recrystallized from water by addition of concentrated HCl.

Preparation of l-Serine and l-Alanine from Silk Fibroin—Technically degummed Japanese white silk (105 gm.) was boiled for 8 hours with 300 cc. of concentrated HCl, the excess HCl removed *in vacuo* in the usual manner, and the bulk of the residual HCl with lead acetate (analytical reagent). The solution was freed of lead with H_2S, and the clear yellow

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[3] Decomposition with $BaCl_2$ is not practicable in this case, since there is not a sufficient difference between the solubilities of the barium and phenylalanine salts of the reagent.

filtrate concentrated *in vacuo* to a syrup. Tyrosine was removed by filtration and the filtrate diluted to about 400 cc.

In this solution 170 gm. of 5-nitronaphthalene-1-sulfonic acid dihydrate were dissolved with heating. The glycine nitronaphthalenesulfonate obtained from the mixture at 0° was recrystallized once from 500 cc. of water, and saved for recovery of the reagent. Yield of recrystallized salt, about 160 gm.

To the hydrolysate remaining after the removal of glycine (volume about 600 cc.) 75 cc. of methyl cellosolve were added and 130 gm. of azobenzene-*p*-sulfonic acid trihydrate were dissolved in the solution with heating. The *l*-alanine salt obtained at 0° was recrystallized once from water[4] (yield, 104 gm.). From this salt both *l*-alanine and the reagent were recovered in the manner described below. The yield of *l*-alanine was about 24 gm., $[\alpha]_D^{26} = +9.5°$ (9.3 per cent amino acid hydrochloride in water). NH_2-N, 15.76 per cent.

To the main body of the hydrolysate a solution of 60 gm. of barium acetate (analytical reagent) was added, and the precipitated barium salts removed. The filtrate was freed of barium with a slight excess of sulfuric acid, and concentrated *in vacuo* to a small volume. The solution was diluted to about 400 cc., 50 cc. of methyl cellosolve and 64 gm. of *p*-hydroxyazobenzene-*p'*-sulfonic acid dihydrate were added, and the mixture heated until a clear solution resulted. The yellow *l*-serine *p*-hydroxyazobenzenesulfonate was obtained at 0°. Some free hydroxyazobenzenesulfonic acid, which is red, may contaminate this precipitate, but it is removed by washing with cold water and by two recrystallizations of the salt from water. Yield of twice recrystallized salt, about 50 gm.

The technique for splitting the amino acid salts and recovering both the amino acid and the reagent is the same in all the instances mentioned here, and will be given in detail only for the case of serine.

To 40 gm. (0.1 mole) of *l*-serine *p*-hydroxyazobenzenesulfonate dissolved in a minimal volume of hot water, a hot solution of 27 gm. (0.1 mole) of barium acetate monohydrate (analytical reagent) was added. The barium sulfonate precipitated immediately but the solution was cooled before filtration. The yellow filtrate, which contained the *l*-serine, was freed of barium with exactly the requisite quantity of sulfuric acid, and acid-washed charcoal added. The water-clear filtrate from the charcoal-$BaSO_4$ was concentrated to dryness *in vacuo*. The residue was dissolved in water, the solution decolorized with charcoal, if necessary, and absolute alcohol added. The serine thus obtained was recrystallized from water and

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[4] Small amounts of inorganic salts of azobenzenesulfonic acid were removed by filtration of the hot solution.

alcohol. Yield, about 8 gm. The amino acid was recrystallized once more for analysis.

$C_2H_7O_2N$. Calculated. C 34.3, H 6.7, N 13.3
Found.   " 34.4, " 6.85, " 13.4

The preparations of *l*-serine yielded by this procedure had a specific rotation of $[\alpha]_D^{20} = -6.8°$ (10 per cent in water) and $[\alpha]_D^{20} = +13.9°$ (250 mg. of *l*-serine plus 2.50 cc. of N HCl). Fischer and Jacobs (6) reported $[\alpha]_D^{20} = -6.8°$ and $[\alpha]_D^{20} = +14.3°$. In determination of the above rotations of serine in N HCl, approximately 1 equivalent of acid was employed. Under these conditions, the specific rotation is sensitive to slight variations in the ratio of serine to HCl. Since it is no longer sensitive to minor variations in the acid concentration when 1.2 or more equivalents of HCl are employed, it appears preferable to determine the rotation in 2 N HCl. $[\alpha]_D^{20} = +14.8°; [\alpha]_D^{20} = +15.3°$ (10 per cent in 2 N HCl).

For recovery of the hydroxyazobenzenesulfonic acid, the barium salt obtained above was heated in a 1:1 water-methyl cellosolve mixture containing a slight excess of sulfuric acid. After removal of the $BaSO_4$, HCl was added to the warm filtrate. The free sulfonic acid crystallized from the cooled solution. For decomposition of barium 5-nitronaphthalene-1-sulfonate, the salt was suspended in aqueous sulfuric acid.

The recovery of the sulfonic acids from their amino acid salts, calculated as per cent of the sulfonic acids originally employed, was 78 per cent for nitronaphthalenesulfonic acid, 73 per cent for azobenzenesulfonic acid, and 52 per cent for hydroxyazobenzenesulfonic acid.

5-Nitronaphthalene-1-sulfonic Acid—The preparation of this substance has been simplified. The crude glycine salt is obtained from the sulfonation mixture in the manner already described (1). For purification, 200 gm. of this salt are suspended in 4 liters of boiling ethanol, and water added until the salt dissolves. The hot solution is clarified with acid-washed charcoal and the pale yellow glycine salt obtained at 0° is washed with ethanol and ether. After one recrystallization from water, the glycine salt is converted to the free acid over the barium salt.

Azobenzene-p-sulfonic Acid (9)—Powdered azobenzene (300 gm.) is slowly added to 900 cc. of fuming sulfuric acid (25 per cent SO_3). The temperature should be kept below 50°. After the azobenzene has dissolved, the solution is heated to 70–80° for a few minutes, cooled to about 50°, and poured into 5 liters of ice. 1 liter of concentrated HCl is added, the mixture cooled to 0°, and the sulfonic acid which separates is recrystallized from water and HCl.

p-Hydroxyazobenzene-p'-sulfonic Acid—The potassium salt is prepared in the manner described by Griess (10). It is converted to the free acid over the barium salt.

SUMMARY

On the basis of the solubility products of their amino acid salts, several sulfonic acids derived from diphenylamine, anthraquinone, and azobenzene have been found to be of potential value for the isolation, purification, and determination of amino acids. The utilization of sulfonic acids for the preparation of amino acids is exemplified by the procedures described for the isolation of *l*-phenylalanine and *l*-leucine from hydrolysates of hemoglobin, and of *l*-serine and *l*-alanine from hydrolysates of silk fibroin.

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SPECIES VARIATION IN NORMAL PLASMA LIPIDS ESTIMATED BY OXIDATIVE MICROMETHODS

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During the past 12 years, beginning with studies under Professor W. R. Bloor (2), the author has analyzed the blood plasma of a number of species of animals, using the same general, oxidative microtechnique throughout. Because the results of lipid analyses vary from method to method and even from person to person using the same method, it was thought that a brief résumé of the "normal" lipid values obtained in the species investigated might be of value. Data previously published have been summarized and a considerable body of new data added.

Accumulated modifications and experience with the oxidative procedure used in this work have been reviewed (5). In this system of analysis, plasma is extracted with Bloor's (1) alcohol-ether mixture at room temperature, due regard being given to the volume of solvent used (5). For the benefit of those who may have adopted the Folch and Van Slyke (8) extraction procedure, it may be noted that extracts prepared by the latter method have yielded, in the hands of the author, lipid values for normal human plasma most of which average 5 to 10 per cent lower than those obtained by dilute cold alcohol-ether at room temperature, as used in all of the results to be reported below. Phospholipid values were found to average 25 to 35 per cent less in the Folch and Van Slyke extracts and, since there was less change in total fatty acids, the calculated values for neutral fat averaged 5 to 10 per cent higher in the Folch and Van Slyke extract.

The values for human (3), cockerel (6), rabbit (4), and guinea pig (7) plasma have been summarized from previous reports as indicated; new data are given for albino rats, cats, and cows. A summary of lipid values in the fasting, oxalated, blood plasma of these seven species of animals is given in Table I. All values are for adult, healthy animals, either males or non-pregnant females.

The diet in these various species consisted essentially of the following: human, standard hospital diet; cockerel, growing mash and whole wheat grain; rabbit, beet pulp and fresh lettuce, cabbage, etc.; guinea pig, the

same as for the rabbit; albino rat, Purina fox chow Checkers and lettuce; cats, milk, table scraps, and meat; cows, pasture.

Of the various lipids, the most constant in each species were the values for total lipid. To estimate the variation, Pearson's coefficient of variation was calculated as the standard deviation divided by the mean and multiplied by 100. The average value of Pearson's coefficient of variation for all the species was 20 in the case of total lipid. Values for neutral fat varied more than those of any other lipid in the majority of the species studied; the average value of Pearson's coefficient for neutral fat was 40. The average coefficient for the remaining lipids was about 30. In other words, about 75 per cent of all normal lipid values for blood plasma will be found to lie within the range of from one-third less to one-third greater

TABLE I

Mean and Standard Deviation of Lipid Values Determined by Oxidative Micromethods in Fasting, Oxalated, Blood Plasma of Normal Animals

The results are expressed in mg. per 100 ml. of plasma.

No. of animals	Guinea pig 10	Albino rat 116	Rabbit 89	Cow 3	Cat 27	Cockerel 22	Man 118
Total lipid .	169±34	230±31	243±89	348±51	376±110	520±85	530±74
Neutral fat	73±33	85±30	105±50	105±39	108± 65	225±77	142±60
Total fatty acids..	116±29	152±23	169±66	202±55	228± 82	361±74	316±85
" cholesterol..	32± 5	52±12	45±18	110±32	93± 24	100±23	152±24
Ester " .	21± 4	31±10	23±12	73±15	63± 23	66±19	106±25
Free "	11± 2	21± 8	22±13	37±15	30± 10	34± 9	46± 8
Phospholipid .	51±12	83±24	78±33	84±21	132± 53	155±34	165±28

than the mean for that species. The variations in rabbits and cats were somewhat greater than this and the variations among the other species somewhat less.

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ASSIMILATION OF HEAVY CARBON DIOXIDE BY HETEROTROPHIC BACTERIA*

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The researches of Wood and Werkman (24) and Wood *et al.* (25, 26) are responsible, in large measure, for the concept of the mechanism of fixation of CO_2 by certain representative genera of heterotrophic bacteria, *Propionibacterium, Escherichia,* and *Citrobacter.* Since investigations in 1938, which demonstrated the relationship of CO_2 fixation to succinate formation, the proposal has been advanced that CO_2 is fixed in a carbon to carbon linkage by C_3 and C_1 addition. Investigations with C^{13} have supported this suggestion in principle. Fixed $C^{13}O_2$ has been found to reside in the carboxyl groups of succinic and propionic acids in accordance with the requirement of C_3 and C_1 addition (26, 28). Carson *et al.* (4) have confirmed this location of fixed CO_2 in propionic acid. Aside from these investigations with propionic acid and coliform bacteria, and with the exception of the investigation of Barker *et al.* (2), studies on the location of assimilated CO_2 in the compounds synthesized by heterotrophic bacteria have not been made. Since such information is essential for an understanding of the mechanism of fixation of CO_2, it is of primary importance that these studies be extended to other bacteria. Therefore, it has been the purpose of this investigation to determine, first the extent of CO_2 assimilation among heterotrophic bacteria, secondly the extent of C_3 and C_1 addition, and thirdly the possibility of assimilation by other mechanisms. As a means of gaining an insight into possible mechanisms of photosynthesis and autotrophism, it is desirable that we have further knowledge of all types of CO_2 fixation, particularly those involving a carbon to carbon linkage.

The results of this investigation show the existence of assimilation of

CO_2 with formation of a *carbon to carbon* linkage by a wide variety of genera of heterotrophic bacteria (*Aerobacter, Proteus, Staphylococcus, Streptococcus, Clostridium*). Fixation of CO_2 by C_3 and C_1 addition is apparently a very general reaction. There is some indication, however, that there may be other mechanisms involved in CO_2 assimilation but the evidence obtained at the present time does not allow definite conclusions to be drawn. Heavy carbon (C^{13}) has been used as a tracer of fixed carbon dioxide.

General Distribution of Fixed Carbon

With the exception of *Clostridium acetobutylicum*, non-proliferating cell suspensions of the respective organisms were used. The majority of experiments were carried out in large manometric flasks of 125 ml. capacity, possessing two side arms. Substrate and $NaHC^{13}O_3$ were placed in the main chamber of the vessel and, in each of two side arms, cell suspension and phosphate buffer (pH 6.3). The vessels were attached to standard Warburg manometers and the air was replaced with oxygen-free nitrogen. Immediately before the manometers were placed in the water bath, the phosphate was tipped into the substrate-$NaHC^{13}O_3$ mixture. The addition of phosphate lowers the pH of the medium to approximately 7.0 and simultaneously carbon dioxide is liberated from the bicarbonate. Thus the acid phosphate insures the presence of a partial carbon dioxide atmosphere above the reaction mixture and also a favorable pH. After equilibration, the cell suspension was tipped into the main chamber of the vessel and the initial reading recorded. When the fermentation, as measured by gas evolution, had ceased, the vessel was removed, and the contents acidified to Congo red with 1:1 H_2SO_4 and centrifuged.

The procedures used to fractionate the fermented media were those employed in this laboratory. In order to determine the C^{13} content of the compounds isolated, it was first necessary to convert them to CO_2. The oxidation was performed according to Osburn and Werkman (19) and the CO_2 evolved collected in 1.5 N carbonate-free NaOH. The CO_2 was then liberated from the alkali, and the C^{13} content determined by mass spectrometer analysis (16). The sodium bicarbonate (4 to 10 per cent C^{13}) was prepared from CO_2 derived from methane, in which the C^{13} had been concentrated in a thermal diffusion column as described by Nier and Bardeen (17).

All naturally occurring materials contain approximately 1.09 per cent C^{13} (18). Inasmuch as the $NaHCO_3$ was the only material in the reaction mixture containing an excess of the carbon isotope, the presence of C^{13} in a compound in excess of 1.09 $\pm$ 0.02 per cent indicates assimilation of CO_2 from the $NaHCO_3$.

TABLE I

Distribution of Assimilated $C^{13}O_2$ among Fermentation Products of Heterotrophic Bacteria

Per cent $C^{13} = \dfrac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100$. The yields of products are expressed as mM per 100 mM of substrate fermented, except *Clostridium acetobutylicum* which is expressed as mM per liter. The bold-faced figures denote assimilation of $C^{13}O_2$.

		Aerobacter indologenes		Streptococcus paracitrovorus		Clostridium welchii	Clostridium aceto-butylicum	Proteus vulgaris	Staphylococcus candidus
Fermentation No		1*	2	3	4	5	6	7	8
Acetic acid	mM. ..	62.7	16.0	137.0	30.8	22.5	34.5	22.7	54.7
	% C^{13}.	**1.33**	**1.19**	1.10	1.11	**1.24**	1.11†	1.10	1.09
Butyric acid	mM .					8.9	33.9		
	% C^{13} .					1.11	1.11†		
Ethyl alcohol	mM	40.3	64.0		68 0	7.7	28.7	18.0	27.6
	% C^{13}.	1.11	1.10		1.08	1.09‡	1.10‡	1.08	1.09
Butyl "	mM .					0.3	41.4		
	% C^{13}					1.09‡	1.10‡		
Acetone	mM						25.7		
	% C^{13}						1.10		
Lactic acid	mM.	1.60		14.3	73.3	10.0	17.1	92.1	20.0
	% C^{13}§ .	**1.36**		1.09	**1.17**	**1.31**	**1.16**	**1.18**	**1.19**
Succinic acid	mM. .	32 0	15.4	21.9	2 4			21.3	11.1
	% C^{13}.	**1.68**	**1.42**	**1.31**	**1.27**			**1.50**	**1.25**
2,3-Butylene glycol	mM		43.0		**27.2**				
	% C^{13}..		1.09		1.09				

* 68 mM of formic acid were also produced which contained 3.13 per cent C^{13}.

† Per cent C^{13} in combined volatile acid distillate.

‡ Per cent C^{13} in combined alcohol distillate.

§ Calculated values; *cf.* Table III.

Fermentation 1—Reaction mixture, 0.125 M glucose, 0.153 M NaHCO₃ (9 per cent C^{13}), 2 per cent cell suspension, volume 60 ml.; incubation period 7 hours at 30°; pH maintained above 7 by addition of NaOH; cells grown for 24 hours at 30° on 1 per cent glucose, 0.3 per cent peptone, 10 per cent tap water.

Fermentation 2—Reaction mixture, 0.1 M glucose, 0.125 M NaHCO₃ (5.29 per cent C^{13}), 0.066 M phosphate buffer pH 6.3, 2 per cent cell suspension, volume 30 ml.; incubation period 10 hours at 30°; growth medium same as Fermentation 1.

Fermentation 3—Reaction mixture, 0.1 M citric acid, 0.0625 M NaHCO₃ (5.29 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 24 hours at 30°; cells grown for 48 hours at 30° on 1 per cent lactose, 0.5 per cent Na₃ citrate, 0.5 per cent peptone, 0.2 per cent peptonized milk, 0.1 per cent yeast extract, 4 per cent filtrate of tomato juice, 0.05 per cent MgCl₂, 0.05 per cent K₂HPO₄, 10 per cent tap water.

TABLE I—*Concluded*

Fermentation 4—Reaction mixture, 0.1 M glucose, 0.0625 M NaHCO$_3$ (4.79 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 30 hours at 30°; cells grown for 48 hours at 30° on medium of Fermentation 3.

Fermentation 5—Reaction mixture, 0.05 M glucose, 0.0625 M NaHCO$_3$ (4.36 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 6½ hours at 40°; cells grown for 20 hours at 38° on 1 per cent glucose, 0.5 per cent peptone, 0.1 per cent yeast extract, 0.5 per cent K$_2$HPO$_4$ adjusted to pH 7 with H$_2$SO$_4$.

Fermentation 6—Reaction mixture, 25 ml. of 5 per cent corn mash, 5 ml. of 0.375 M NaHCO$_3$ (10.64 per cent C^{13}), 2 ml. of 0.2 M phosphate buffer pH 6.3; inoculum consisted of 2 ml. of 24 hour corn mash culture; bicarbonate and phosphate added 5 hours after inoculation; incubation period 2½ days at 38°.

Fermentation 7—Reaction mixture, 0.05 M glucose, 0.0625 M NaHCO$_3$ (4.36 per cent C^{13}), 0.02 M phosphate buffer pH 6.3, 3 per cent cell suspension, volume 30 ml.; incubation period 30 hours at 30°; cells grown for 24 hours at 30° on 1 per cent glucose, 0.3 per cent beef extract, 0.5 per cent peptone, 0.4 per cent NaCl, 2 per cent agar.

Fermentation 8—Reaction mixture, same as for Fermentation 7; incubation period 7 hours at 30°; cells grown for 48 hours at 30° on 1 per cent glucose, 0.3 per cent beef extract, 0.3 per cent peptone, 0.2 per cent NaCl, 0.2 per cent K$_2$HPO$_4$.

The matter below Table I presents the exact composition of the various reaction mixtures, growth media, etc. The cells were harvested from the respective media by centrifugation, washed once, and resuspended in distilled water. Cell suspensions of *Clostridium acetobutylicum* were not found satisfactory as regards production of neutral compounds (acetone, alcohols); so that a growing culture in 5 per cent corn mash containing NaHC^{13}O$_3$ was used.

Table I indicates the distribution of the assimilated CO_2 among the fermentation products of the various bacteria. Two species of homofermentative lactic acid bacteria, *Streptococcus lactis* and *Lactobacillus plantarum*, were found to fix no CO_2. It is interesting to note that such compounds as 2,3-butylene glycol, butyric acid, and butyl alcohol, which are probably formed by a synthesis, contained no fixed CO_2.

Fixation of Carbon Dioxide in Succinic Acid

The degradation of succinic acid (26) was performed by a succinic dehydrogenase-fumarase enzyme[1] prepared from beef heart according to Krebs (10). Succinate, obtained as the silver salt from the fermented medium, was acidified, extracted with ether, and then converted to the sodium salt. The reaction mixture consisted of 30 ml. of approximately 0.02 M sodium succinate, 0.04 M phosphate buffer (pH 7.4), and 10 ml. of

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[1] Thanks are expressed to G. Kalnitsky for the preparation of this enzyme.

enzyme preparation.   The experiments were performed aerobically at 30°
in large manometric flasks.   The oxidation products, malate and fumarate,
were oxidized with permanganate to CO_2 and acetaldehyde and the latter
then oxidized to CO_2 with persulfate.

Table II shows that succinate formed by *Aerobacter, Streptococcus*, and
Proteus contained fixed carbon in the carboxyl groups.   Wood *et al.* have
also found a similar fixation of CO_2 in the C_4-dicarboxylic acids with *Esch-
erichia* and *Propionibacterium* and by pigeon liver (27).

The following reactions were proposed by Wood *et al.* (26) to explain
the synthesis of succinate.

$$C^{13}O_2 + HCH_2 \cdot CO \cdot COOH \rightleftharpoons C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH$$
$$C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH + 4H \rightleftharpoons C^{13}OOH \cdot CH_2 \cdot CH_2 \cdot COOH + H_2O$$
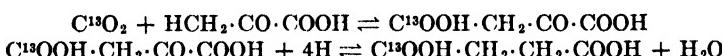
It is probable that the same or similar reactions are involved in the present
study.   Further experimentation is needed to prove definitely the exact

TABLE II

Position of Assimilated $C^{13}O_2$ in Succinic Acid Synthesized by Heterotrophic Bacteria
The values are given in per cent C^{13}.

Bacteria	Succinic acid	α- and β-carbon	COOH carbon	Calculated* COOH carbc
Streptococcus paracitrovorus.. .	1.27	1.10	1.36	1.45
Proteus vulgaris	1.50	1.12	1.82	1.91
Aerobacter indologenes.. . .	1.42	1.13	1.61	1.75
" " . .	1.37	1.14	1.84	1.61

* Calculated by means of the equation, $2 \times 1.09 + 2X = 4 \times$ per cent C^{13} in suc-
cinic acid; X = average per cent C^{13} in carboxyl groups of succinic acid.

intermediate steps in the C_3 and C_1 addition but it is not unlikely that
oxalacetate is an intermediate.   The results recently obtained by Krampitz
and Werkman (9) and unpublished results with the aid of C^{13} particularly
support this suggestion.

In most of the experiments the concentration of C^{13} was not determined
in the $NaHCO_3$ and CO_2 at the conclusion of the fermentation.   However,
in one experiment with *Aerobacter indologenes* (Table I, Fermentation 1)
in which this determination was made, the C^{13} content of the final $NaHCO_3$
plus gaseous CO_2 contained 3.13 per cent C^{13}; the succinic acid formed
contained 1.68 per cent.   On the basis that all fixed carbon in succinate
resides in one carboxyl group, the calculated value for this carboxyl carbon
is 3.45 per cent C^{13}.   Assuming the per cent C^{13} fixed in one carboxyl group
should be approximately equal to the per cent C^{13} of the final CO_2, a large
part of the succinic acid is indicated to arise in this fermentation by C_3
and C_1 addition.   On the contrary, in the fermentations by *Staphylococcus
candidus, Streptococcus paracitrovorus,* and *Proteus vulgaris,* the calcula-

tions of C^{13} content of final CO_2 are too high to indicate a stoichiometric fixation of CO_2 by C_3 and C_1 addition. This variation in the C^{13} content of succinate may indicate that in addition to the fixation mechanism there is yet another mechanism of succinate formation not involving fixation. The relative proportion of succinate formed by these two mechanisms may vary and thus cause the observed variation in the C^{13} concentration of the succinate. There is of course the possibility that such variation is caused by difference in cell permeability to CO_2 from one experiment to another; *i.e.*, the amount of C^{13} available to the cell for fixation may not be the same in similar experiments.

It is interesting that the succinate contained fixed carbon in every case in which it was formed. This indicates that formation of succinic acid by fixation of CO_2 is a general reaction. It does not necessarily follow, however, that this is the only mechanism of succinate formation.

Fixation of Carbon Dioxide in Lactic Acid

Slade *et al.* (22) showed for the first time the fixation of CO_2 in lactic acid by various heterotrophic bacteria. Wood *et al.* (27) have also found a similar fixation in lactate produced in the dissimilation of pyruvate by pigeon liver. In order to ascertain the position of the assimilated carbon in the lactate molecule, the permanganate oxidation method of Friedemann and Graeser (6) was used. Acetaldehyde and CO_2 are end-products of this oxidation. The aldehyde originates from the α- and β-carbon groups and CO_2 from the carboxyl group of the lactic acid molecule. The CO_2 was collected in carbonate-free alkali and the aldehyde trapped in 2 per cent $NaHSO_3$. Calcium carbonate was added to the solution of the aldehyde-sulfite complex, and the liberated aldehyde distilled into ice-cold water. Barium hydroxide was then added to the distillate and the precipitated carbonates filtered off. The aldehyde was then oxidized to CO_2.

In every case in which fixation occurred in lactate the fixed carbon was found in the carboxyl group (Table III). Of the number of possible mechanisms for this fixation, either C_2 and C_1 addition or a secondary conversion of a C_4-dicarboxylic acid containing fixed carbon in the carboxyl group seems most probable. Lactic acid is generally accepted to be formed by reduction of pyruvic acid; so that any scheme that accounts for fixation of CO_2 in the carboxyl group of pyruvic acid provides a probable source of lactic acid containing fixed carbon in the carboxyl group. The exchange of CO_2 with the carboxyl group of pyruvic acid through action of carboxylase seems a likely mechanism for fixation of the heavy carbon. Thus far, however, all experimental evidence indicates that decarboxylation of pyruvic acid by carboxylase is irreversible. This point has been investigated both by Evans and Slotin (5) by use of C^{11} with yeast carboxylase and by Krampitz (unpublished data) with C^{13} and an acetone preparation

of *Micrococcus lysodeikticus* which oxidatively decarboxylates pyruvate to acetate and CO_2. There was no evidence of reversibility of this reaction. Nevertheless, it was shown that such an experimental procedure is a reliable one for proving the existence of exchange reactions involving carboxyl carbon and CO_2. By the use of C^{13} and the enzyme preparation which converts oxalacetate to pyruvate and CO_2 (9), the presence of a reversible exchange in this reaction was proved. Although an investigation with the bacteria used in this work is to be desired, it seems fairly certain that CO_2 fixed in lactic acid does not result from the action of carboxylase.

The alternative mechanism, *i.e.* by formation of lactate from a C_4-dicarboxylic acid, offers more attractive possibilities but there are at present

TABLE III

Position of Assimilated $C^{13}O_2$ in Lactic Acid Synthesized by Heterotrophic Bacteria
The values are given in per cent C^{13}.

Bacteria	Lactic acid*	COOH carbon	α- and β-carbon
Staphylococcus candidus	1.19	1.34	1.11
Streptococcus paracitrovorus	1.17	1.33	1.09
Clostridium welchii	1.31	1.68	1.13
" *acetobutylicum*	1.16	1.22	1.13
Proteus vulgaris	1.18	1.34	1.11
Aerobacter indologenes	1.36	1.81	1.13

* Calculated by means of the equation, $2 \times$ per cent C^{13} in α,β-carbons + per cent C^{13} in carboxyl carbon = $3 \times X$; X = per cent C^{13} in lactic acid molecule.

certain unexplained facts. The following series of reactions serves to illustrate a possible mechanism.

$$C^{13}O_2 + CH_3 \cdot CO \cdot COOH = C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH = C^{13}OOH \cdot CH{:}COH \cdot COOH$$
$$= C^{13}OOH \cdot COH{:}CH \cdot COOH = C^{13}OOH \cdot CO \cdot CH_3 + CO_2 \underset{-2H}{\overset{+2H}{\rightleftharpoons}}$$
$$C^{13}OOH \cdot CHOH \cdot CH_3 + CO_2$$
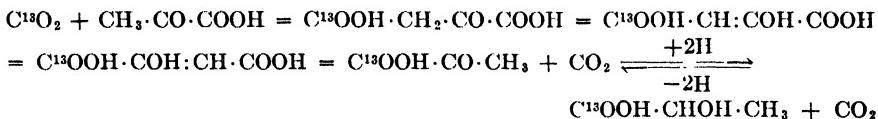
The scheme incorporates the suggestion of Meyerhof (12) that there exists a dynamic, non-enzymic interchange of H and OH between the α- and β-carbons of enol oxalacetic acid. This reaction causes an equal distribution of the C^{13} in both carboxyl groups of the oxalacetic acid. There is as yet no direct experimental evidence for this equilibrium. Carson *et al.* (4) have proposed the reversible conversion of oxalacetate to the symmetrical molecule, fumarate, as a possible mechanism to explain the formation of pyruvic acid containing fixed carboxyl carbon. Such a mechanism would lead to formation of succinate containing fixed carbon in both carboxyl groups, since part of the heavy carbon pyruvate would again condense with $C^{13}O_2$ and be converted to succinate. In the present experiments and

those of Wood *et al.* (25), the C^{13} values have never indicated a fixation of more than 1 molecule of $C^{13}O_2$ per molecule of succinate.

The following are some observations which at first sight may seem inconsistent with the above theories. Lactic acid formed from glucose by *Streptococcus paracitrovorus* contained fixed carbon, whereas that from citrate did not. On the other hand the succinate from both fermentations contained fixed carbon (*cf.* Table I). It follows that the lactate was formed from citrate by a mechanism which differs from that responsible for the formation of C^{13} lactate from glucose. Assuming oxalacetate containing fixed carbon was formed in both fermentations and C^{13} lactate was formed in the glucose fermentation from oxalacetate, there must have been a mechanism present in the citrate fermentation which prevented breakdown of oxalacetate to yield heavy carbon pyruvate. Moreover, in other fermentations there does not seem to be any relationship between the concentration of C^{13} in the carboxyl groups of succinate and lactate, as might be expected if both had a common source. This variation may result either because there is more than one source of succinate or because oxalacetate may be removed prior to its breakdown to pyruvate more completely in some fermentations than in others. *Clostridium welchii* produced lactic acid containing heavy carbon but there was no C_4-dicarboxylic acid formed. In this fermentation it would be necessary to assume all the C_4 acid was broken down to compounds containing fewer carbon atoms.

Two species of homofermentative lactic acid bacteria (*Streptococcus lactis, Lactobacillus plantarum*), *i.e.* bacteria producing substantially only lactic acid, were examined and in none was there fixation of CO_2. Some significance was at first attached to this finding in that these bacteria have a weak carboxylase activity. It has been pointed out previously that pyruvate carboxylase probably has no connection with fixation of CO_2. It now seems more likely that these bacteria are unable to accomplish C_3 and C_1 addition and hence cannot form heavy carbon lactate.

Our views on the mechanism of fixation of CO_2 in lactate are speculative. The scheme pictured above does not assign an essential rôle to the fixation; *i.e.*, lactate would be formed regardless of CO_2 fixation. It is conceivable that the formation of lactate by the fixation reaction may occur by a path in which fixation is an essential step but yet independent of the non-fixation mechanism. In this case the reaction would be of fundamental importance.

Fixation of Carbon Dioxide in Acetic Acid

The results of Slade *et al.* (22), showing the fixation of CO_2 in acetic acid produced by *Aerobacter* and *Clostridium*, present the important problem of ascertaining the position of the assimilated carbon in the molecule.

The procedure used was similar to that of Ardagh *et al.* (1).   Wood *et al.* (28) have proved the reliability of this method to determine the position of fixed CO_2 in propionic acid.   After recovery of the acetic acid from the fermented medium, purity was checked by the partition method of Osburn *et al.* (20).   In the presence of brom-thymol blue, $Ba(OH)_2$ was added to neutrality and the solution evaporated to dryness.   The dry barium acetate was placed in a 25 ml. distilling flask and held at 460° in a Wood's alloy metal bath for 50 minutes.   The flask was continuously flushed with oxygen and CO_2-free nitrogen.   The reaction is as follows:

$$(CH_3COO)_2Ba \rightarrow CH_3 \cdot CO \cdot CH_3 + BaCO_3$$
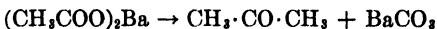
The carbonate originates from the carboxyl group of acetate.   Acetone was collected in ice-cold water.   The CO_2 was liberated from the residual carbonate by addition of 2 N HCl.

The acetone was distilled from the original solution and then converted to iodoform (8) by treatment with successive aliquots of NaOH and I_2. The solution was allowed to stand $\frac{1}{2}$ hour in an ice bath; a slight excess of H_2SO_4 was then added, and the liberated I_2 titrated with $Na_2S_2O_3$.   Iodoform was filtered onto a sintered glass disk, dried over $CaCl_2$ at room temperature, and oxidized to CO_2 with chromic-sulfuric acid (7).

The yields of CO_2 from the dry distillation ranged between 90 and 95 per cent of theoretical, and acetone 45 and 50 per cent.   The recovery of iodoform is practically quantitative.

In order to secure sufficient acetate to perform the degradation, experiments with *Aerobacter* and *Clostridium* were performed in a volume of 60 ml., containing 0.1 M glucose, 0.06 to 0.15 M $NaHC^{13}O_3$, and 2 per cent cell suspension under N_2.   Mickelson and Werkman (14) have shown that large yields of acetate result from glucose by *Aerobacter* when the fermentation is kept above pH 6.3.   Dilute NaOH in the presence of brom-thymol blue was added during the incubation period.   A previous experiment had shown that CO_2 fixation in acetate occurred under alkaline as well as acid conditions.

In the case of *Clostridium welchii*, it was necessary to separate a mixture of acetic and butyric acids before the former acid could be degraded.   The benzene distillation method of Schicktanz *et al.* (21) was used.   A 3 foot still packed with porcelain saddles was employed.   After distillation, acetic acid was recovered from the benzene solution by alkaline evaporation, acidification with H_2SO_4, and subsequent steam distillation.   The purity of the acetic acid was checked according to the method of Osburn *et al.* (20).

The residue containing butyric acid, benzene, and *p*-toluenesulfonic acid was filtered, water added, and the mixture neutralized and evaporated to small volume.   After a second filtration the solution was acidified and

steam-distilled, and the distillate neutralized, evaporated to small volume, acidified, and again steam-distilled. The partition constant of the distillate indicated the presence in addition to butyric acid, of traces of an unknown acid. The distillate, after oxidation, was not found to contain fixed C^{13} (cf. Table I).

Barker et al. (2) using a little known organism, Clostridium acidi-urici, were able to show by the aid of radioactive carbon, C^{11}, the reduction of CO_2 to acetic acid in the presence of uric acid. The CO_2 was found in both methyl and carboxyl groups. Wieringa with Clostridium aceticum (23) has also shown a reduction of CO_2 to acetic acid with gaseous hydrogen as a reducing agent.

The type of fixation in acetic acid to be described here differs fundamentally from that noted in the above examples in that the assimilated CO_2 is present only in the carboxyl group. Table IV shows that in two

TABLE IV

Position of Assimilated $C^{13}O_2$ in Acetic Acid Synthesized by Heterotrophic Bacteria

The values are given in per cent C^{13}.

Bacteria	Acetic acid	Methyl carbon	Carboxyl carbon	Calculated* carboxyl carbon
Aerobacter indologenes.........	1.33	1.11	1.34	1.57
" "	1.17	1.10	1.19	1.23
Clostridium welchii....	1.13	1.11	1.15	1.15

* Calculated by means of the equation, $1.09 + X = 2 \times$ per cent C^{13} in acetic acid; $X =$ per cent C^{13} in carboxyl group of acetic acid.

experiments with Aerobacter indologenes the carboxyl carbon contained 1.34 and 1.19 per cent C^{13}, whereas conversely the methyl carbon contained 1.11 and 1.10 per cent C^{13} respectively. Likewise, with Clostridium welchii carboxyl carbon contained 1.15 per cent C^{13}, methyl carbon 1.11 per cent C^{13}. The per cent CO_2 fixed in acetate by the latter organism has been consistently lower when compared to Aerobacter. In a single experiment these values would be questionable but from repeated experiments it is concluded that none of the fixed carbon is located in the methyl group. In an additional experiment with Clostridium welchii, acetic acid contained 1.24 per cent C^{13} and the acetone derived therefrom contained 1.19 per cent. Calculating all the fixed C^{13} to reside in the carboxyl carbon of acetate, a value of 1.19 per cent is obtained for acetone which agrees with the experimental value. In this experiment the CO_2 from the carboxyl group was lost.

The amount of carbon fixed in acetic acid has been quite variable and is not as high as that obtained in lactate and succinate; nevertheless the

values are significant.   Acetic acid is generally believed to arise by oxidation of pyruvic acid.

$$CH_3 \cdot CO \cdot COOH + H_2O \rightarrow CH_3 \cdot COOH + CO_2 + 2H$$
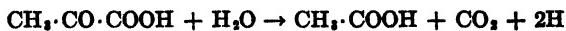
In this reaction the carboxyl carbon is transformed to CO_2; therefore even though there is fixed carbon in the carboxyl group of pyruvate, the resulting acetic acid would be devoid of fixed carbon.   It thus is apparent that there are two mechanisms responsible for the formation of acetic acid, one in which the acetate contains fixed carbon, the other in which it does not. This fact may explain the variation in the amount of C^{13} fixed.   The fixation reaction may not be directly concerned in the formation of acetic acid, in that the acetic acid may arise by secondary conversions following the initial fixation; e.g., by C_3 and C_1 addition.   The possibility of fixation of C^{13} in acetic acid by an exchange reaction seems remote.   If such a reaction did occur it would involve a new type reaction of fundamental importance; i.e., C_1 and C_1 addition in which one component is not CO_2 or formed from CO_2.   A more likely mechanism is that initial fixation takes place by the Wood and Werkman reaction and the acetate is subsequently derived from the C_4-dicarboxylic acid by splitting of the molecule into two C_2 fragments.   Such a reaction, if reversible, would be of great importance and would perhaps indicate the oxidation of acetic acid by way of the C_4-dicarboxylic acids.   Krebs and Eggleston (11) in experiments with *Propionibacterium shermanii* fail to consider this in explaining an increased yield of succinate on addition of acetate to oxalacetate fermentations.   The oxidative condensation of acetate to succinate may take place anaerobically with the participation of oxalacetate as a hydrogen acceptor. An increase in succinate on addition of acetate to oxalacetate fermentations has likewise been noted in this laboratory with *Citrobacter*.

The possibility also exists of a separation of a C_2 fragment from an intermediate compound which may contain more than 4 carbon atoms.   Such a scheme would, of course, be required to explain the observed fixation.   For example, the acetic acid may be separated from one of the 6- or 7-carbon intermediate compounds that occur in the modified Krebs cycle (27).

Reference to Table I will show that with *Aerobacter*, succinic acid is an end-product, while with *Clostridium welchii* no succinate is formed; yet acetate contains fixed CO_2.   This fact does not necessarily invalidate the participation of the C_4 acids in the formation of acetate by this organism.

Acetate may also arise by α decarboxylation of oxalacetate to malonic aldehyde, followed by oxidation and subsequent decarboxylation.

$$C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH \xrightarrow{-CO_2} C^{13}OOH \cdot CH_2 \cdot CHO \xrightarrow[-2H]{+H_2O}$$

$$C^{13}OOH \cdot CH_2 \cdot COOH \longrightarrow C^{13}OOH \cdot CH_3 + CO_2$$
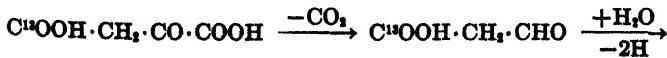
Little evidence is available to support this scheme; however, aerobic experiments have shown the oxidation of malonic acid by *Aerobacter*.

It is of interest to note that Mickelson and Werkman (15) have shown the reduction of propionic acid to propyl alcohol by *Aerobacter* and have suggested that a similar reaction takes place with acetic acid. No evidence of such a reaction was found in these experiments, since ethyl alcohol recovered contained no fixed C^{13}.

Mickelson (13) also found a cell-free, non-volatile, non-ether-soluble residue from alkaline fermentations of glucose by *Aerobacter*. This material has not been identified but is known not to contain adjacent hydroxyl groups. In one experiment the cell-free residue remaining after steam distillation and ether extraction was found to contain fixed carbon (1.24 per cent C^{13}). Barker *et al.* (3) in experiments with cell suspensions of *Methanobacterium* in the presence of $C^{11}O_2$ have found fixed carbon in the fermentation residue which contained cell material and probably other non-volatile substances. Barker *et al.* believe these results to show the incorporation of CO_2 into cell material. The present results indicate the possibility that the fixation may have been in other than cell material.

SUMMARY

The assimilation of CO_2 is established as a general phenomenon among heterotrophic bacteria. It is shown by the use of heavy carbon, C^{13}, as a tracer, that the fixed carbon is located in the carboxyl groups of succinic, lactic, and acetic acids. The assimilated CO_2 is distributed as follows: *Aerobacter indologenes*, acetate, lactate, and succinate; *Proteus vulgaris*, *Streptococcus paracitrovorus*, and *Staphylococcus candidus*, lactate and succinate; *Clostridium welchii*, acetate and lactate; *Clostridium acetobutylicum*, lactate.

Succinic acid contained fixed carbon in every case that it was formed. Fixation of CO_2 in succinic acid is believed to arise by C_3 and C_1 addition according to the Wood and Werkman reaction. Conversely, lactate and acetate did not contain fixed carbon in all cases. Thus, there are fundamental differences in the mechanism of formation of these compounds among heterotrophic bacteria. Possible mechanisms are discussed.

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THE ACID-SPLITTING REACTION OF ACETOACETIC ACID AND THE ENZYMATIC FORMATION OF ACETIC ACID FROM ACETOACETIC ACID*

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There is very little direct chemical evidence on the mechanism of the breakdown of the ketone bodies in the extrahepatic tissues. Among the several theories offered for the catabolism of acetoacetic acid, it has often been suggested (1–3) that it undergoes hydrolysis to 2 molecules of acetic acid prior to further catabolism. However, it has never been demonstrated that this reaction can take place either by enzymatic or non-enzymatic catalysis under any conditions approaching the physiological.

It was the purpose of this investigation to study the conditions, kinetics, and catalysis of the hydrolysis in purely chemical systems *in vitro*, and then to determine whether any chemical data can be obtained for the existence of an enzyme or enzymes which bring about a formation of acetic acid from acetoacetic acid in excised tissues, tissue extracts, or microorganisms.

EXPERIMENTAL

Confirmation and Extension of Previous Data—Although Wislicenus (4) and von Euler and Ölander (5) had previously studied the hydrolysis of acetoacetic acid by strong bases, a complete analysis of the reaction has not been reported. It seemed desirable to confirm and extend certain of their data.

Sodium acetoacetate solutions were prepared throughout this study by the method of Ljunggren (6). In the experiments reported in Table I the classical Messinger-Huppert method (7) was used for the determination of acetoacetic acid, the method of Folin (8) for acetone, and the methods of Virtanen and Pulkki (9) for acetic acid identification and determination.

The data on the course and kinetics of ketone- and acid-splitting at various concentrations of sodium hydroxide generally confirm those of von Euler and Ölander and need not be discussed further. However, it can be seen from the data (Table I) that acetic acid was the only volatile acid formed in the reaction, that it accounted for all of the hydrolyzed aceto-acetic acid, and that no side reactions occurred. This last observation was unexpected and is important.

Effect of Possible Catalysts on Rate of Acid-Splitting Reaction—Since no catalyst other than strong bases has been reported for the acid hydrolysis, a series of experiments was then set up as in Table I, with various compounds

TABLE I

Hydrolysis of Sodium Acetoacetate by Sodium Hydroxide at 37°

The initial concentration of acetoacetate is approximately 0.069 M in each case. The changes in the reactants are expressed in mM.

Experiment No.	NaOH concentration	Time	Initial acetoacetate	Acetoacetate hydrolyzed	Acetic acid formed	Recovery
	N	*hrs.*				*per cent*
1	0.1	24	17.01	All hydrolyzed to acetone		
2	3	18	16.95	8.92	16.76	99.5
3	3	24	16.95	10.01	19.90	99.1
4	5	24	17.05	12.38	24.60	99.2
5	8	12	17.60	8.72	17.34	·99.2
6	11	48	16.93	16.02	31.80	99.3

Identification of Acid by Duclaux Constants

	Per cent volume distilled			
	25 per cent	50 per cent	75 per cent	87.5 per cent
Unknown....	16.94	36.30	59.20	73.80
Pure acetic acid	16.91	36.32	59.16	73.85

substituted for the sodium hydroxide as possible catalysts for the reaction. The compounds and concentrations used (necessarily selected somewhat at random) were 3.5 N NH_3, 1 M Na_3PO_4, 1 M Na_2HPO_4, 3 M NH_4Cl, saturated NH_4Cl, saturated NaCl, 0.1 M $HgSO_4$, 1 per cent HgO (freshly precipitated), 1 N $FeSO_4$, 1 per cent colloidal $Fe(OH)_3$, alumina gel, 1 per cent colloidal $Al(OH)_3$, 10 per cent $Ca(OH)_2$ suspension, 10 per cent $Cu(OH)_2$, 1 per cent freshly precipitated Ag_2O, 1 per cent sodium silicate, silica gel, 1 per cent MnO_2 suspension, and the common buffer mixtures (bicarbonate, phosphate, glycine, acetate, borate). In no case could any breakdown of acetoacetate into acetate be established.

Another series of experiments was then performed in which the solutions

of acetoacetic acid were buffered at pH 7.4 by 0.05 M phosphate buffer and the possible catalytic effect of a series of compounds known to be important in cellular metabolism was then determined. The concentration of aceto-acetate was uniformly 0.01 M. Acetoacetic acid was determined by the method of Messinger-Huppert but in cases in which interference with the method was expected the results were checked by means of the standard Van Slyke procedure (10). The compounds examined were succinate, malate, fumarate, oxalacetate, citrate, α-ketoglutarate, pyruvate, lactate, dl-alanine, aspartic acid (all in 0.01 M concentration), 0.005 M l-cysteine, 0.1 per cent H_2O_2, 0.01 M dl-glyceraldehyde, 0.005 M sodium hexose mono-phosphate, 10 mg. per cent cytochrome (crude preparation from beef heart), 0.01 M glucose, 0.01 M acetaldehyde, 0.01 M hydroquinone, 20 units per 100 ml. of insulin (Lilly's iletin), 1 per cent hemoglobin, and the common physiological anions and cations in 0.01 M concentration. There was no measurable decrease in acetoacetic acid in any case.

Enzymatic Formation of Acetic Acid from Acetoacetic Acid—Since it seemed unlikely from the results of these simple experiments that aceto-acetic acid is hydrolyzed to acetic acid by a non-enzymatic catalysis other than by strong alkalies, the possibility of an enzymatic catalysis of this reaction was then investigated. This search for an enzyme was begun with minced rabbit muscle, and this was the preparation studied in the following series of experiments.

Technique and Methods

The reaction vessels were 1 liter Erlenmeyer flasks. 10 gm. of minced rabbit muscle (Latapie mill) were added to 100 ml. of medium (Krebs-Ringer-phosphate buffer of pH 7.4 (11) containing the neutralized sub-strate) which had been previously equilibrated at 38°. The appropriate gas was bubbled through the mixture for 10 minutes; the flask was then closed off, and shaken in a thermostat bath at 38° for 2 hours. Initial and final filtrates were prepared for analysis. In all operations involving acetoacetic acid, care was exercised to prevent any possibility of excess alkali causing hydrolysis to acetic acid artificially.

Acetoacetic acid and β-hydroxybutyric acid were determined on aliquots of copper-lime filtrates by Edson's micro modification of Van Slyke's method (12). In some experiments the acetoacetic values were checked by Dewan and Green's modification (13) of the manometric aniline citrate method, with the standard Warburg apparatus.

The determination of the very small amounts of volatile fatty acid formed in the experiments offered some difficulties. For the analysis of small samples (5 ml.) containing not less than the equivalent of 0.5 ml. of 0.01 N NaOH the method described by Leloir and Munoz (14) was found to be

satisfactory and was used when acetate was the substrate. However, in the determination of smaller amounts of acetate in large volumes of buffer the method is unsatisfactory, since the solutions must first be concentrated by alkaline evaporation before distillation, and hydrochloric acid (from buffer chlorides) was found to be present in the distillates from such concentrated filtrates, rendering the determinations worthless.

It was found possible to eliminate these difficulties by steam-distilling the concentrated tissue filtrate, by use of the apparatus and reagents described by Friedemann (15); 300 ml. of the distillate were collected. This distillate contains hydrochloric acid and the fatty acid to be estimated and is made slightly alkaline and evaporated down to 30 ml. on a water bath and the distillation repeated with the addition of 0.05 gm. of freshly prepared Ag_2O to the reagents used in the distillation. The distillate is caught in 25 ml. fractions in Erlenmeyer flasks, boiled for 7 to 8 seconds, and titrated with 0.01 N NaOH from a micro burette with phenolphthalein. After the fifth fraction has been collected, the titration value usually has dropped to that of a water-reagent blank distillation, which is performed beforehand and which is subtracted from the titration value of each fraction. The total of the corrected titration values represents the total volatile fatty acids.

This method was found to be capable of determining as little as 30 γ of acetic acid in a 200 ml. volume of buffer with 10 per cent accuracy and larger quantities with much greater accuracy. All fractions were tested after titration with silver nitrate and barium chloride for the presence of chloride and sulfate.

Variation in Recovery of Acetic Acid from Tissues—In some recovery experiments it was found that the added acetate could not be completely recovered. To determine the method of deproteinization which allowed best recovery some experiments were designed to measure recovery of acetate when added to muscle mince and immediately treated to remove proteins. In these experiments acetate was added to 100 ml. of buffer containing 10 gm. of rabbit muscle mince, filtrates prepared as indicated, and analyses of acetic acid performed. See Table II.

Apparently acetate is bound to some extent by tissue proteins and is not removed completely even in the acid Folin-Wu and phosphotungstic acid procedures. The binding of acetic acid by tissue proteins has also been observed by Dr. E. M. MacKay (personal communication) and by Stadie, Zapp, and Lukens (16). This recovery error must be taken into account in any quantitative consideration.

Identification of Residual Endogenous Fatty Acids in Tissue Filtrates—In the experiments reported later under the formation of steam-volatile fatty acids, there was a small titratable acidity on the distillation of the

initial filtrates.   To determine the nature of these acids the initial value distillates of Experiments 1 to 7 (Table V) were pooled and treated as in the previous section.   Duclaux distillation yielded the constants given in Table III.

From the data it can be seen that acetic acid and possibly a trace of a less steam-volatile acid make up the endogenous volatile fatty acids encountered in the blank controls and the initial value experiments.

Inhibition of Acetate Utilization—Since acetate is rapidly utilized by most tissues (2), some means of preventing this utilization must be employed if

TABLE II

Recovery of Added Acetate from Rabbit Muscle Mince

10 gm. of mince in 100 ml. of buffer containing 10.0 micromoles of acetate were used in each case.   The recoveries of acetate are reported in micromoles.

Experiment No.	Treatment	Recovery
1	Copper-lime, pH 8.3	8.4
2	Same, pH 10.5	7.3
3	Folin-Wu	8.9
4	Saturated $HgCl_2$	7.6
5	" phosphotungstic acid	7.0
6	Theoretical recovery	10.0

TABLE III

Duclaux Constants of Endogenous Volatile Fatty Acids of Rabbit Muscle Mince

	Per cent volume distilled			
	25 per cent	50 per cent	75 per cent	87.5 per cent
Unknown	17.4	37.3	63.1	77.6
Pure acetic acid .	16.6	36.2	59.7	74.6

the formation of acetic acid from acetoacetic acid is to be demonstrated. Some experiments with malonate, arsenite, fluoride, selenite, etc., as inhibitors showed that both acetoacetate and acetate breakdown were inhibited almost equally, making them worthless for this purpose.   However, it was found that the utilization of acetate depends on the oxygen tension, and that when the gas phase is air instead of oxygen the utilization of acetate is depressed, while that of acetoacetate remains essentially unchanged from that in pure oxygen (see Table IV).   Thus, with air as the gas phase, the most likely conditions for obtaining acetoacetate breakdown and retarding acetate breakdown are realized.

Formation of Steam-Volatile Fatty Acids from Acetoacetic Acid—With air

as the gas phase, experiments were then performed to determine whether any steam-volatile acids were formed when acetoacetic acid was utilized,

TABLE IV

Effect of Oxygen Tension on Utilization of Acetoacetate and Acetate by Rabbit Muscle Mince

10 gm. of rabbit muscle mince suspended in 100 ml. of buffer containing added acetoacetate or acetate in the concentrations given below were shaken for 2.0 hours at 38° as described under "Technique and methods." The figures below represent the utilization of the metabolite in terms of micromoles per 10 gm. of mince per 2 hours. In the case of acetoacetate they represent true utilization and do not include conversion to β-hydroxybutyric acid, which was also measured but not included in this table.

| | Concentration | Gas phase | | |
		Oxygen	Air	Nitrogen
	micromoles per 100 ml.			
Acetoacetate	1000	120	140	None
	500	85		
	80	19	14	None
Acetate	1000	230	90	"
	500	155		
	80	42	15	5

TABLE V

Formation of Steam-Volatile Fatty Acid from Acetoacetic Acid by Rabbit Muscle Mince

The gas phase was air, and the incubation period was 2 hours at 38°. The data are reported in micromoles.

Experiment No.	Initial aceto-acetic acid	Acetoacetic acid reduced to β-hydroxybutyric acid	Disappeared acetoacetic acid	Acid formed	Per cent aceto-acetic acid re-covered as acid
1	80.6	12.7	11.3	8.4	37
2	80.6	12.1	21.4	41.0	96
3	81.0	12.9	15.1	11.4	38
4	81.0	13.1	None	None	0
5	81.1	14.3	9.4	7.1	38
6	86.0	17.1	None	None	0
7	89.0	14.2	17.6	4.4	12
8	74.0	12.0	12.4	8.4	34
9	93.0	9.4	13.1	2.1	8
10	76.4	18.3	None	None	0

according to the technique described. The volatile acid figures are calculated as acetic acid. Blank control experiments were run in each case, and the figures in Table V are corrected for the small blank experiment changes.

Parallel experiments in every case on autoclaved tissue and tissue treated with 0.01 N NaCN and 0.005 M HgCl₂ showed no utilization of acetoacetate, indicating that the action is probably enzymatic in nature. The presence of 40 units of insulin or 0.01 M glucose or both had no noticeable effect on the transformation.

For the identification of the acid formed, the distillates of the final filtrates from seven experiments were pooled, made slightly alkaline, and evaporated to 50 ml. The acids were then distilled as previously described, giving a total of about 4 ml. of 0.01 M acid. A portion of the sample yielded a positive lanthanum reaction (17), indicating the presence of acetic acid. The other portion was then subjected to a Duclaux distillation, yielding the data given in Table VI.

The Duclaux constants show that the acid formed from acetoacetic acid is mainly acetic acid, with possibly a trace of a less steam-volatile acid, probably formic.

TABLE VI

Duclaux Constants of Unknown Acid Formed from Acetoacetic Acid by Muscle Mince

	Per cent volume distilled			
	25 per cent	50 per cent	75 per cent	87.5 per cent
Unknown	17.4	37.2	62.0	77.0
Pure acetic acid	16.6	36.2	59.7	74.6
Fraction 2 of unknown redistilled.	17.4	37.1	57.3	76.0

In considering the data in Table V it is quite apparent that the results show an extremely great variation both in amount of acetoacetate metabolized and in amount of acetate formed. Several factors may be enumerated as being at least partly responsible. First, the analytical methods are not very accurate or specific (the Edson method is described as being accurate only to within 10 per cent and it is known from his work that pyruvate, if present, interferes considerably; the acetate determination is accurate to only 10 per cent and is also subject to a loss in recovery as described). Second, it is possible that there are variations in the metabolic state of the tissue. Also, the inherent difficulties of the excised tissue technique must be considered.

However, although the data are not quantitatively reproducible from animal to animal, they show qualitatively that acetate is produced only when acetoacetate disappears and that these changes do not appear when the tissue is autoclaved or treated with cyanide or mercuric chloride.

In order to confirm this suggestion attempts were then made to obtain the enzyme free of the cells.

Formation of Acetic Acid by Homogenized Tissue Preparations—Muscle mince-buffer suspensions homogenized by the technique of Potter and Elvehjem (18) and kidney mince ground with sand and 0.05 M phosphate buffer at pH 7.4 and then centrifuged to remove insoluble cell material showed a formation of volatile acid as acetoacetate disappeared. This acid was likewise identified as acetic acid. With the kidney preparation the results were readily reproducible although the changes were small. Both preparations retained an active oxygen uptake and metabolized acetic acid.

The formation of a volatile acid by the kidney preparation confirms Quastel and Wheatley's (19) finding of an increased number of acid groups, as acetoacetate was utilized by kidney slices in bicarbonate buffer.

TABLE VII

Formation of Steam-Volatile Fatty Acid from Acetoacetate by Extracts of Muscle and Kidney

The incubation period was 1 hour; temperature, 38°. The data are reported in micromoles and the fatty acid figures are calculated as acetic acid.

Extract	Initial acetoacetate	Final acetoacetate	Acetic acid formed	Per cent acetoacetate recovered as acetic acid
Muscle................	80.0	69.9	5.2	23
"	81.2	70.2	4.5	21
"	76.5	62.0	8.4	29
No substrate	1.1	1.8	None	
Kidney.........	81.0	68.0	7.1	27
"	81.0	67.4	7.0	26
"	81.0	69.0	6.4	27
No substrate.	1.0	0.6	0.2	
Substrate + 0.01 M NaCN... ..	81.0	83.7	0.4	
Extract heated to 100°..	81.0	85.3	0.4	

These data, then, confirm the suggestion of the previous mince experiments that an enzymatic formation of acetic acid from acetoacetic acid can occur.

Many extracting procedures and media were used without success in attempts to prepare a more active enzyme concentrate. A cofactor may possibly be necessary, since well washed muscle mince did not give the reaction nor was the wash water (when concentrated *in vacuo*) alone active. The enzyme when obtained in homogenized form was very unstable and became inactive in 1 hour at 38° and 3 hours at 0°, rendering further purification difficult. The data are given in Table VII.

Formation of Acetic Acid from Acetoacetic Acid by Escherichia coli—In the search for a biochemical catalyst, a few bacterial cultures were tried.

It was found that pure cultures of *Escherichia coli* produced acetic acid from acetoacetic acid under anaerobic conditions on a synthetic medium, on peptone, and on an autoclaved kidney extract, the reaction being of much greater extent than in the tissue experiments just described.

The synthetic medium employed was composed of 0.1 per cent K_2HPO_4, 0.1 per cent $(NH_4)_2SO_4$, 1.0 per cent $CaCO_3$, and 0.5 per cent sodium acetoacetate. The peptone medium was composed of 0.2 per cent peptone, 0.2 per cent $(NH_4)_2SO_4$, 25 per cent 0.25 M phosphate buffer (pH 7.0), and 0.5 per cent sodium acetoacetate. The kidney extract was prepared by homogenizing 100 gm. of minced beef kidney in 1 liter of the Krebs-Ringer

TABLE VIII

Formation of Acetic Acid from Acetoacetic Acid by Escherichia coli

The incubation period was 24 hours at 38°. The gas phase was 95 per cent N_2, 5 per cent CO_2. The data are given in micromoles.

Experiment No.		Initial acetoacetate	Final acetoacetate	Acetic acid formed	Per cent acetoacetate recovered as acetic acid
1	Synthetic medium + acetoacetic acid	840	97	725	49
	" " + " "	842	90	522	35
	" " + " "	856	110	543	37
2	" " without acetoacetic acid	0	0	0	
3	" " + 0.01 M NaCN	856	833	0	
4	" " + 0.001 M $HgCl_2$	856	863	0	
5	Kidney extract + acetoacetic acid	160	32	251	98
	" " + " "	170	34	174	64
6	" " without acetoacetic acid	0	4	4	
7	Peptone + acetoacetic acid	880	210	970	73
8	" without acetoacetic acid	12	22	42	

buffer, centrifuging, and autoclaving the supernatant. The media were set up with the usual sterile precautions and inoculated with a loopful of a saline suspension of *Escherichia coli*. Blank control experiments were set up to determine the endogenous acid production. Data are given in Table VIII.

The acid formed was subjected to a Duclaux distillation and the constants found were identical with those of pure acetic acid.

Control experiments with an equivalent amount of acetone as substrate showed only a very slight formation of acetic acid, showing that acetone was probably not an intermediate in the reaction.

These results on the anaerobic metabolism of acetoacetic acid by *Escherichia coli* supplement those of Deotto (20) who found that aerobically

acetate was not an intermediate in the metabolism of acetoacetic acid by this organism.

The data on the kidney extract cultures show that, when the organism has another source of carbon, acetate is not attacked, and quantitative recovery of 2 molecules of acetic acid from 1 of acetoacetate was possible. In these experiments large amounts of acetoacetate are utilized and the more reliable macromethods can be used. These experiments conclusively show that an acid hydrolysis of acetoacetic acid is a possible cellular process.

DISCUSSION

Although the data here presented on the muscle mince experiments are admittedly not quantitatively significant, the qualitative observation that acetic acid may be formed from acetoacetic acid is well established by the experiments on the homogenized preparations and the bacterial cultures. These data show that this reaction can take place in certain cellular processes and provide the first experimental basis for postulating such a reaction for the breakdown of acetoacetate.

Although it has often been suggested that acetoacetic acid is hydrolyzed to 2 molecules of acetic acid in the extrahepatic tissues, there has been no direct chemical evidence for such an assumption. Rumpf (21) found that diabetics excreted as much as 5 gm. of acetic acid in the urine per day. This may have originated by such a reaction or may have been an end-product of fatty acid oxidation in the liver, although Stadie et al. (16) were unable to show any formation of acetic acid by excised liver slices from depancreatized cats.

The results here reported show a formation of acetic acid from aceto-acetic acid which is small and irregular but definite. The mechanism of this formation is not clear from the experiments but three possibilities exist: (1) an actual hydrolysis into 2 molecules of acetic acid; (2) a phosphorolysis into acetic acid and acetylphosphoric acid; and (3) formation of acetic acid by dismutation of pyruvic acid, which may be formed from acetoacetic acid, according to the suggestions of Hoff-Jorgensen (22). In view of the evidence here presented these theories of acetoacetic acid catabolism should be retained until more chemical evidence is obtained on this long standing problem.

Very little is known about the metabolism of acetic acid. From the work of MacKay et al. (23) it is known that the phlorhizinized dog converts acetic acid into ketone bodies, but this reaction is believed to take place only in the liver and not in the extrahepatic tissues. It is more probable that acetic acid is utilized in extrahepatic tissues by some other mechanism.

All attempts to reverse this reaction in muscle mince met with failure. Although the results obtained with tissues and bacterial cultures indicate

the presence of an enzyme capable of forming acetic acid from acetoacetic acid, nothing can be said at present about the physiological importance of this transformation, except that it must be included in any consideration of the catabolism of acetoacetic acid until further evidence is obtained.

The author wishes to express his appreciation for the advice and interest of Professor E. J. Witzemann.

SUMMARY

1. Acetic acid was the only product obtained when acetoacetic acid was hydrolyzed by strong bases. No other catalyst could be found for the reaction.

2. Rabbit muscle mince formed small quantities of acetic acid from acetoacetic acid, when incubated at 38° for 2 hours.

3. Pure cultures of *Escherichia coli* formed large quantities of acetic acid anaerobically from acetoacetic acid.

4. The catalytic system involved in the muscle mince and the bacteria was thermolabile and possessed other classical properties of an enzyme.

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SPECTROPHOTOMETRIC DETERMINATION OF IRON

II. USE OF 2,2'-BIPYRIDINE

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Since its introduction by Hill (1) as a reagent for iron, 2,2'-bipyridine (α,α'-dipyridyl) has been used in numerous colorimetric determinations of this element (2–4). The sensitivity of the test makes it particularly suitable for the determination of iron in foods and biological materials, and the extreme stability of the ferrobipyridine ion (5, 6) renders the method relatively free from interferences. Since an accurate procedure for spectrophotometric use does not seem to have been developed, the present work was undertaken for this purpose.

Apparatus and Reagents—The apparatus and the preparation of most of the reagents and standard solutions used in this research are described in Paper I of this series (7). Additional reagents were prepared as follows:

A phthalate buffer of pH 3.6 was made by dissolving 10.21 gm. of recrystallized, centrifugally drained, and vacuum-dried potassium acid phthalate in water, adding 59.7 ml. of 0.1000 N hydrochloric acid, and making to a liter. An acetate buffer of pH 3.6 contained 60.0 ml. of glacial acetic acid and 10.00 gm. of recrystallized and suitably dried sodium acetate trihydrate in a liter. The chromogenic agent was a 0.200 per cent solution of 2,2'-bipyridine.

Attempts to use purified sodium thiosulfate or sodium hydrosulfite for the reducing agent, as specified in many colorimetric procedures, showed the improbability of obtaining accurate results with either of these compounds. Even after careful purification they release colloidal sulfur on reducing ferric iron, and under many conditions reduction is slow or incomplete. These reducing agents were eventually abandoned as wholly unsatisfactory.

A 1 to 2 per cent solution of sulfur dioxide was found to be a very convenient and satisfactory reducing agent. This solution was prepared by passing tank sulfur dioxide through two wash bottles containing respectively concentrated sulfuric acid and cotton and collecting the gas in a beaker of distilled water on a trip balance. To determine the exact concentration before any series of analyses a 1.00 ml. portion of solution was

pipetted into 50 ml. of water, 1 ml. of starch solution was added, and the mixture was titrated with 0.05 N iodine to a blue color.   The 0.05 N iodine was made by dissolving 6.346 gm. of sublimed iodine in 150 ml. of water containing 10 gm. of potassium iodide and making to a liter.   1 ml. of this solution is equivalent to 0.00160 gm. of sulfur dioxide.   A sulfur dioxide solution kept in the paper-covered Pyrex reservoir of an automatic burette exposed to ordinary daylight over a period of 2 months showed in the iodine titrations only slight variations in reducing power, gave clear solutions on reducing ferric iron, and produced color development reproducible to 1 part in 250 during the entire time.

Summary of Calibration Experiments

Calibrations were made by the procedure described for analyses, except that the test solutions contained accurately measured quantities of iron, and the concentrations of acids, bipyridine reagent, and other factors were varied systematically to find permissible, adequate, or optimum quantities. Measurements were made at temperatures from 29–31°.   Different combinations of primary standards and reagents were used, to eliminate constant errors.   Early in the work it was shown that the pH of test solutions may vary between 2.5 and 5.0 without significant color variations, but most measurements were made at pH 3.6.

The median transmittances found in about 700 observations made with 175 test solutions are recorded in Table I.   These comprise the readings taken 12 to 24 hours after color development, and represent the attainment of constant color.   The 12 to 24 hour medians were used in calculating the average deviations both for the 30 minute and 12 to 24 hour observations (systems containing trichloroacetic acid or perchloric acid being excluded) and the deviations thus show the relative trustworthiness of readings taken at these times.   Transmittance readings made 30 minutes after color development were frequently the same as the 12 to 24 hour readings, occasionally too high, but rarely low.   This is evidently due to slow formation of the pink complex ion.   It follows that the occasional high transmittances for 30 minute readings may to some extent be avoided by postponing dilution to final volume.   Also, observations made in another connection indicate that heating the systems to 90° for 5 minutes, followed by cooling and dilution to volume, will improve the accuracy of 30 minute readings provided interfering effects such as colloid formation or oxidation of the complex ion are avoided.   However, it is usually convenient to let test solutions stand 12 to 24 hours at room temperature before readings are taken.   This is especially desirable when the solutions contain phosphate or pyrophosphate, as is often the case in analyses of foods or biological materials.

Transmittances for systems without added acid agreed closely enough with those for systems with added acid to show that acids in concentrations up to those specified in the procedure below have no significant effect on the color. Smallest deviations were obtained with no added acid, 5 ml. of 6 N hydrochloric acid, or 2 ml. of 36 N sulfuric acid. Addition of 0.1 ml. of 30 per cent hydrogen peroxide did not interfere with color development, but when 0.5 ml. was added transmittances were too high. It is thus best to destroy peroxide by boiling the analytical solution before the various reagents are added.

No significant differences were observed when the phthalate and acetate buffers were used interchangeably, or in the few cases in which iron-free sodium or potassium hydroxide was substituted for ammonium hydroxide.

<div align="center">

TABLE I

Concentration-Transmittance Data for Iron by Bipyridine

</div>

Fe per 100 ml.	Transmittances (12–24 hrs.)		Average deviation of single observations	
	Blank reference	Water reference	30 min.	12–24 hrs.
mg.	*per cent*	*per cent*	*per cent*	*per cent*
0.0400	83.1	82.1	0.3	0.2
0.1000	63.6	62.6	0.6	0.4
0.200	40.5	39.6	1.4	0.5
0.300	25.8	25.1	3.5	0.7
0.400	16.4	15.9	4.4	0.8
0.500	10.3	10.0	4.0	1.7

Spectrophotometric Procedure for Total Iron

The following procedure is based on the experiments described above, and has been further tested by comparison with similar procedures in which ammonium mercaptoacetate and ferron were used as chromogenic agents, in numerous analyses of foods and biological materials.

Procedure

Ash (3) a measured sample (1 to 20 gm.) and make it up to a suitable volume (50 to 250 ml.) after hydrolyzing any pyrophosphate present (3, 8, 9). Take for analysis an aliquot containing not more than 0.50 mg. of iron. If a wet ashing method was used, evaporate the aliquot to dryness and destroy organic matter by heating the residue in succession with 0.5 ml. portions of 36 N sulfuric acid and 30 per cent hydrogen peroxide, or by electrical heating. · Take up the residue with 5 ml. of 6 N hydrochloric acid, dilute to 20 ml., and reflux for 30 minutes to hydrolyze pyrophosphate.

In all cases treat the aliquot to remove any interfering inorganic sub-

stances (3) known to be present, if maximum accuracy is desired. The solution for analysis may contain up to 10 ml. of 6 N hydrochloric acid, 2 ml. of 36 N sulfuric acid, or 40 ml. of 0.6 N trichloroacetic acid. Up to 5 ml. of 9 N perchloric acid may be present if the solution contains less than 0.20 mg. of iron; with higher concentrations a red precipitate forms after the bipyridine is added.

Add the equivalent of 4 ml. of 1 per cent sulfur dioxide solution to the iron solution and allow it to stand for 5 minutes. Add a small piece of Congo red paper and 4 N ammonium hydroxide until the paper has the same color as a similar piece in a buffer of pH 3.6. Add 10 ml. of acetate or phthalate buffer of pH 3.6, then 10 ml. of 0.2 per cent bipyridine reagent. Allow the undiluted solution to stand at least 20 minutes; then dilute to exactly 100 ml. For best results, and always when trichloroacetic acid, perchloric acid, or nitric acid is present, make duplicate transmittance readings 12 to 24 hours later on two portions of the solution, at a wavelength of 510 mμ. Calculate the result of the analysis by substituting the median value of T in the proper equation, as indicated below, or by use of a graph made from the equation.

The analytical solution may also contain up to 1 ml. of 9 N perchloric acid together with 5 ml. of 15 N nitric acid. In this case neutralize the solution *before* adding 1 per cent sulfur dioxide solution and use 8 ml. of this reagent. Then neutralize again, add the buffer, and proceed as described.

If the reagents are free from iron, use water as the reference liquid; otherwise use a blank containing the reagents. If a colored aliquot is used without removing the color, use as a reference liquid a system containing no bipyridine reagent and the same volume of colored liquid as the test solution.

Beer's law holds and data in Table I have been reduced by the method of least squares to a pair of linear equations, in the first of which,

$$\text{Mg. Fe per 100 ml. test solution} = \frac{-0.664 \log_{10} T + 1.329}{l} \qquad (1)$$

T is the percentage transmittance relative to a blank, and l is the thickness of the solution in cm. In the second equation, with water as the reference liquid, the constants are respectively -0.658 and 1.313. In the present calibration l was 1.308 cm. The actual value of l should be determined for the cuvettes actually used, with a micrometer and calipers.

SUMMARY

An accurate spectrophotometric method for the determination of iron with 2,2'-bipyridine has been developed experimentally.

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A LOW TEMPERATURE WET ASHING METHOD APPLIED TO THE STUDY OF THE ELECTROLYTE COMPOSITION OF THE VENTRICULAR MUSCULATURE AND LUNG PARENCHYMA OF THE DOG*

By EARL H. WOOD†

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Dry ashing is the standard method for the preparation of organic material for electrolyte analysis; hence this method has been used in most investigations of the electrolyte metabolism of soft tissues. The chief disadvantages of the method are (1) that it requires relatively expensive apparatus (platinum crucibles and muffle furnace) and (2) that there is danger of volatilization of significant amounts of some electrolytes when the digestion temperature is kept high enough to yield a white ash.

Wet ashing of organic material requires less expensive apparatus and there is little danger of volatilization of electrolytes. The usual oxidizing agents employed are sulfuric, perchloric, and nitric acids, hydrogen peroxide, and their various combinations. The disadvantages which are responsible for the limited use of this method arise from the fact that the final solution is either very strongly acid, so that large amounts of base are required to neutralize the excess acid, or the digestion must be continued over a relatively hot flame to drive off the excess acid. Volatilization of sulfuric or perchloric acid over an open flame is a difficult procedure to carry out without occurrence of creeping or spattering resulting in partial loss of the sample, unless a relatively large and subsequently disadvantageous digestion tube is used. If only relatively volatile oxidizing substances, such as nitric acid (Harrison *et al.* (1)) and hydrogen peroxide which can be removed by heating on a water bath, are used, a colored, incompletely oxidized, residue results which is not completely soluble and consequently difficult to work with.

In a study of electrolyte metabolism of heart muscle which has been carried out in this laboratory (Wood and Moe (2, 3))[1] a wet ashing method

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[1] Wood, E. H., and Moe, G. K., to be published.

has been developed which avoids the difficulties mentioned above. The entire digestion is completed on a water bath and results in a colorless, crystalline-like residue which is readily water-soluble and contains relatively small amounts of acid.

Methods

Potassium analyses were carried out by a modification of the silver cobaltinitrite method (Wood).[2] Sodium was determined by a modification of the uranyl zinc acetate method of Barber and Kolthoff (4). The method of Van Slyke and Sendroy (5) was used for chloride determinations.

The heart and lungs of ether-anesthetized dogs were removed at death following hemorrhage. The ventricles were separated from the atria by cutting along the atrioventricular groove. The visible fat and connective tissue were trimmed from the ventricles and the remaining relatively fat-free ventricular musculature run twice through a meat chopper. The lung parenchyma was treated similarly after visible bronchi had been trimmed off flush with the parenchyma. Approximately 2 gm. duplicate samples of the minced ventricular musculature and lung parenchyma were weighed out in 25 × 100 mm. Pyrex test-tubes. The samples were dried to constant weight in an oven kept at 95–100° and the water content determined by weight differences.

The dried tissue samples were wet-ashed in the same Pyrex tubes by the following procedure: Approximately 2 cc. of concentrated nitric acid are added for each gm. of fresh tissue sample. The tubes are placed on a boiling water bath and 1 or 2 drops of caprylic alcohol added at intervals during the first 30 minutes of digestion to reduce foaming. After about 10 hours on the water bath a small quantity of a dry yellowish residue remains. Approximately 2 drops of a sulfuric-perchloric acid mixture are then added per gm. of fresh tissue. This solution, as described by Leulier and Bernard (6), is a mixture of 65 to 35 parts by volume of concentrated sulfuric and 60 per cent perchloric acids respectively. After addition of this mixture the partially digested residue chars in from 10 to 30 minutes. 6 drops of 30 per cent hydrogen peroxide are added. The solution clears for a time but gradually darkens again. The addition of hydrogen peroxide is repeated several times until the residue remains as nearly colorless, moist crystals. This requires several hours, depending upon the amount and character of the tissue. 6 cc. of distilled water are added to the residue which readily dissolves, forming a clear slightly greenish tinted solution. A drop of 0.1 per cent phenolphthalein in alcohol is added and sufficient powdered calcium hydroxide to make the solution basic to this indicator. The phosphates precipitate out as calcium phosphate. The solution is

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[2] Wood, E. H., to be published.

well agitated and then poured into a 15 cc. centrifuge tube, stoppered, and centrifuged for 5 minutes.

2 cc. of the supernatant fluid are pipetted into a sodium-precipitating tube, acidulated by addition of a drop of dilute acetic acid, and evaporated to approximately half volume in a desiccator or oven. The sodium content of this aliquot sample is determined by direct precipitation in this tube as the uranyl zinc acetate complex.

1 or 2 cc. aliquot samples of the supernatant fluid are pipetted into 15 cc. Pyrex test-tubes which are placed in an oven at 100° and evaporated to dryness. If the residue does not remain pink, it is necessary to add 1 or 2 cc. of distilled water and sufficient calcium hydroxide to make the solution again alkaline to phenolphthalein and repeat the evaporation. This procedure removes the ammonia. 10 or 15 cc. of distilled water are added to the pink dry residue; the tube is stoppered and agitated until the residue is completely dissolved. Duplicate aliquot samples corresponding to 0.05 to 0.1 gm. of heart muscle or about 0.20 mg. of potassium are pipetted from this solution into pointed tip, calibrated, Pyrex centrifuge tubes, the volume made up to 5 cc., and potassium precipitated as the silver potassium cobaltinitrite complex. These aliquot samples are chloride-free, since the chloride is volatilized during the acid ashing procedure.

Blood samples were heparinized or defibrinated, centrifuged at 3000 R.P.M. for 30 minutes, and the supernatant plasma or serum withdrawn from the cells with a pipette and reserved for analysis.

Results

Water, potassium, sodium, and chloride analyses have been carried out on the ventricles, lungs, and blood serum of a series of normal dogs. The average and extremes of the values obtained expressed on a wet and dry weight basis are given in Table I. Calculations, based upon the averages of the above analyses, have been made as described by Hastings and Eichelberger (7) to obtain a value for the "extracellular water"[3] in normal dog ventricles.

The calculated "extracellular water" values from the chloride analyses and sodium analyses are 23.2 and 24.6 gm. per 100 gm. of fresh ventricular musculature respectively. Adopting the "extracellular water" value of 23.2 gm. per cent as a measure of the extracellular phase of the ventricles, we have calculated the water and potassium content of the intracellular phase. The results of these calculations are summarized in Table II. The com-

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[3] "Extracellular water" has been used (8) as a non-committal term for the calculated chloride space, since this calculated value is actually neither the "chloride space" nor "extracellular space" but only a good approximation of these two phases of tissue.

<div align="center">TABLE I</div>

Tissue Analyses of Normal Dogs Killed by Hemorrhage

The water content is measured in gm.; the potassium, sodium, and chloride concentrations are given in mM.

	Water content per 1000 gm. fresh tissue	Potassium		Sodium		Chloride	
		Per 1000 gm. fresh tissue	Per 100 gm. dry weight	Per 1000 gm. fresh tissue	Per 100 gm. dry weight	Per 1000 gm. fresh tissue	Per 100 gm. dry weight
Ventricles							
No. of dogs...........	18	18	18	11	11	11	11
Average........	783	81.5	37.4	35.3	16.3	29.0	13.4
Extremes.............	766–	73.4–	31.6–	32.7–	14.3–	24.8–	11.7–
	799	87.0	40.0	39.1	18.9	33.0	15.6
Lung parenchyma							
No. of dogs...... ...	15	16	16	12	12	10	10
Average.....	786	51.0	23.9	78.1	36.5	62.2	29.0
Extremes..	771–	40.0–	19.3–	67.8–	31.2–	54.6–	24.2–
	801	64.1	31.6	91.6	42.9	71.9	34.4
Blood plasma							
No. of dogs...........	19	19		18		16	
Average....	937	4.56		142		113	
Extremes.............	926–	3.50–		138–		104–	
	940	5.70		145		124	

<div align="center">TABLE II</div>

Comparison of Electrolyte and Water Composition of Cardiac Muscle with Striated Muscle of Normal Dogs

			H₂O per kilo fresh tissue	Potassium	Sodium	Chloride
			gm.	mM per kg. H₂O	mM per kg. H₂O	mM per kg. H₂O
Ventricles	Plasma		937	4.87	151	120
	Whole ventricles		783	104	45.2	37.1
	Extracellular space	(23.2 gm.%)	990	4.74	145	127
	Intracellular space	(76.8 gm. %)	721	145	3.77	0.0
Striated muscle (rectus abdominis) (from Hastings and Eichelberger (7))	Plasma		922	4.03	154	119
	Whole muscle		765	107	42.3	28.1
	Extracellular space	(17.3 gm. %)	990	3.90	148	126
	Intracellular space	(82.7 gm. %)	717	137	11.0	0.0

position of normal striated muscle (rectus abdominis) as given by Hastings and Eichelberger (7) is included in Table II in comparison with ventricular cardiac muscle.

The electrolyte and water composition of these two types of muscle appears to be very similar. The chief point of difference and probably the only significant divergence which is evident from Table II is in the relative magnitudes of the respective intra- and extracellular phases. The extracellular phase of cardiac muscle per unit of fresh muscle tissue appears to be considerably larger than the extracellular phase of striated muscle. This finding appears logical in the light of the known histology and physiology of these tissues.

Owing to the probable presence of intracellular chloride, and the difficulty in correcting for blood contained in lung tissue, no attempt has been made to calculate the extracellular phase of lung tissue.

DISCUSSION

The average values for the electrolyte composition of normal dog plasma and ventricular musculature which are reported in this paper are in excellent agreement with similar values published recently from other laboratories (Muntwyler *et al.* (9); Darrow *et al.* (10)).

A wet ashing method has recently been reported by Buell (11) which employs nitric acid, followed by a perchloric-sulfuric acid mixture without the use of hydrogen peroxide.

SUMMARY

1. A low temperature wet ashing method for biological materials is described. The digestion can be carried to completion on a water bath and produces a readily water-soluble colorless ash. In essence the method consists of a preliminary digestion with nitric acid, followed by a perchloric-sulfuric acid mixture, the digestion then being completed with hydrogen peroxide.

2. The averages and ranges of values for potassium, sodium, chloride, and water content of the ventricular musculature, lung parenchyma, and blood serum of a series of normal dogs are reported.

3. The "extracellular water" content and the "intracellular" electrolyte and water composition of the ventricular musculature have been calculated.

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MONOLAYERS OF COMPOUNDS WITH BRANCHED HYDROCARBON CHAINS

V. PHTHIOCEROL

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Phthiocerol is a higher optically active alcohol of the formula $C_{34}H_{67}(OH)_2OCH_3$ or $C_{35}H_{69}(OH)_2OCH_3$ which Stodola and Anderson (1) have isolated from the wax of the human tubercle bacillus. It is a constant and characteristic constituent of the wax of the human (2) and bovine (3) tubercle bacillus but has not been found in other acid-fast bacteria. The optical rotation $[\alpha]_D$ is $-4.8°$ and the melting point 73–74°. The hydrocarbon derived from phthiocerol melts at 58.5–59.5° and the formula $C_{34}H_{70}$ gives the best agreement with analysis.

The distribution of the polar groups and the structure of the hydrocarbon chain are as yet unknown and the experiments reported below were undertaken in the hope of getting some information on these points. Professor Anderson kindly put a specimen of phthiocerol at our disposal.

EXPERIMENTAL

The technique used has been described in Paper I (4) and Paper IV (5) of this series.

Monolayers[1]

The results obtained with phthiocerol spread on a 0.01 N HCl substrate at different temperatures are shown in Fig. 1. It forms a typical "liquid expanded" (6) or "expanded mesomorphous" (7, 8) monolayer.

The limiting area of the fully expanded monolayer is 122 sq. Å. at 20° and 134 sq. Å. at 34°. At 5° the monolayer is solid condensed. The smallest area to which the monolayers can be compressed before collapse sets in is 30 to 31 sq. Å. The temperature of half expansion (Adam (6)) at 1.5 dynes pressure is about 17°. In the transition region *b–c* (Fig. 1) a certain amount of hysteresis is present but otherwise the mono-

[1] For the calculation of area values, the $C_{35}H_{72}O_3$ formula (mol. wt. 540) has been used. For the $C_{36}H_{74}O_3$ formula (mol. wt. 554) the area values should be multiplied by 1.026.

layers are fairly stable and reproducible. When condensed (at c), the monolayers are solid. The curves in Fig. 1 were obtained on rapid compression starting 3 minutes after spreading. The same limiting areas were found whether the compression was started 1 or 10 minutes after spreading.

The spreading of phthiocerol on 0.01 N HCl substrate at 15° was also controlled by ultramicroscopical examination with the Leitz "ultropak."[2] The normal ring condenser, objective 11 ×, and ocular 10 × were used
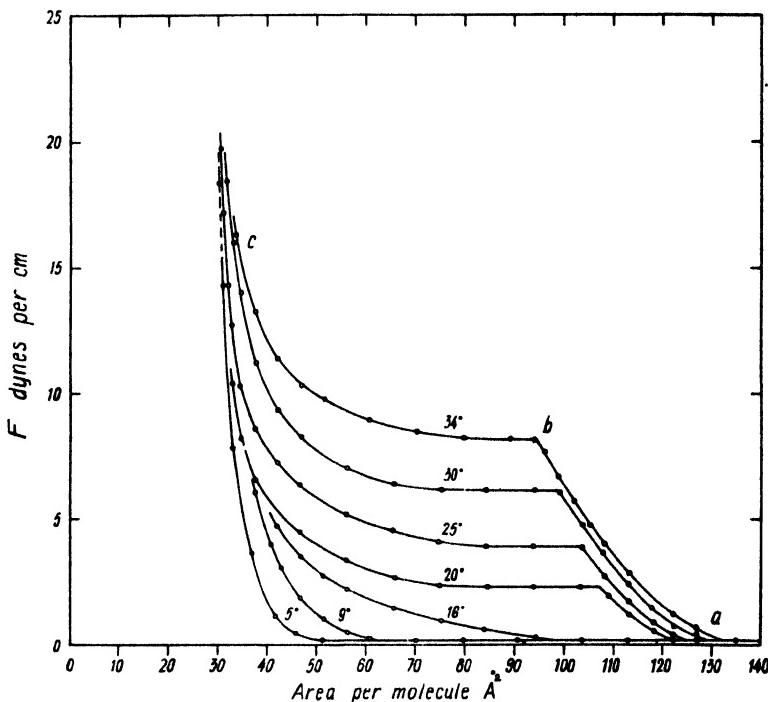
FIG. 1. Force-area curves for phthiocerol spread on 0.01 N HCl at different temperatures.

(total magnification 110 ×). The dark-field was completely empty, indicating proper spreading, up to a pressure of 13 dynes (about 32 sq. Å. per molecule). Above this pressure there appeared, on rapid compression, a very fine "point structure" (9), which disappeared on standing or on expansion. At higher pressures the point structure became more clearly visible. A phthiocerol monolayer which had been compressed

[2] The use of this instrument for the ultramicroscopical investigation of monolayers will be described in detail by one of us (S. S.) in a forthcoming paper.

beyond the point of collapse showed no formation of streaks parallel to the compressing barrier.

Fig. 2 shows the surface potential-area and apparent surface moment-area curves given by phthiocerol when spread on 0.01 N HCl at 20°. The surface potential is 285 millivolts at the limiting area and increases to about 510 millivolts at the point of collapse. No fluctuations greater
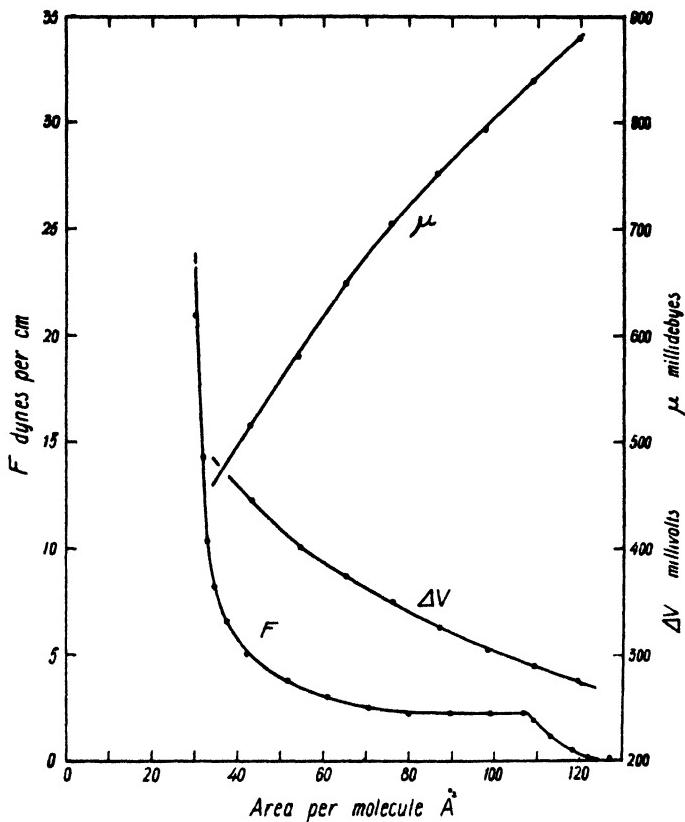
FIG. 2. Force-area, surface potential-area, and surface moment-area curves for phthiocerol on 0.01 N HCl at 20°.

than 6 to 10 millivolts were found in the transition region *b–c*. The apparent surface moment is very high, 880 millidebyes at 122 sq. Å., and falls on compression to about 450 at 31 sq. Å.

As phthiocerol contains no polar group capable of undergoing ionization, practically identical force-area curves are obtained on substrates of different pH, at corresponding temperatures.

Attempts to Build Multilayers and x-Ray Experiments

By use of the Langmuir-Blodgett technique (10, 11) attempts were made to build multilayers of phthiocerol on chromium-plated slides. A large number of different substrates (distilled water, 0.01 N HCl, 5 N HCl, distilled water containing 40 per cent ethyl alcohol, tap water) at temperatures from 5–50° and piston pressures from 16 to 35 dynes were tried, but, as the monolayers of phthiocerol in the condensed state are very stiff under all these conditions, only poor results were obtained. With tap water as substrate and triolein as piston (spreading pressure 23 dynes) at room temperature it was found, however, that if the slide was lowered very slowly through the monolayer deposition did not occur on the down journey but occurred on the up journey of the slide (Z deposition (12)). A twenty-five layer film obtained in this way was not optically perfect, but had an optical thickness corresponding to about forty layers of standard barium stearate. The optical thickness per layer of the phthiocerol multilayer, assuming the refractive indices of both films to be equal and the optical thickness per layer of standard barium stearate to be 24.4 Å. (11), was about 39 Å.

In order to measure the long x-ray spacing, the twenty-five layer Z-deposited multilayer was subjected to x-ray analysis by the technique described previously (5, 12).[3] In spite of very long exposures (up to 36 hours), no lines corresponding to a definite long spacing were obtained and it was evident that the phthiocerol multilayer was very poorly crystalline.

DISCUSSION

For the expanded part of a liquid expanded film (*a–b* in Fig. 1) Langmuir (14) has given the following equation of state

$$(F - F_0)(a - a_0) = kT$$

where

F = surface pressure observed in dynes per cm.
a = area observed in sq. Å.
F_0 and a_0 = empirical constants
k = gas constant in two dimensions
T = temperature in °K.

Normal chain compounds, such as myristic acid, obey the above equation very well. The constants F_0 and a_0 depend on the structure of the molecules.

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[3] A more detailed x-ray investigation of phthiocerol is in progress. In a note published after this paper had been written, Spiegel-Adolf and Henny (13) state that phthiocerol gives a complicated diffraction pattern but report no long (001) spacing.

For phthiocerol the equation

$$(F + 6.0) (a - 65) = kT (F_0 = -6.0; a_0 = 65)$$

agrees with the part a-b in Fig. 1 of the force-area curve at 34°. It appears, however, that F_0 and a_0 are not independent of temperature, as the agreement at 20° is not so good.

The interpretation of the surface experiments in terms of molecular structure is in the case of phthiocerol difficult owing to the lack of material for comparison. The fact that it forms a typical liquid expanded monolayer with a smallest stable area of 30 to 31 sq. Å. suggests that phthiocerol is essentially a very long molecule with one or more of the polar groups in a position at or very near one end, and, if side chains other than the methoxyl group are present, that these are short, probably methyl. Apart from this, very little can be said with any degree of certainty. No information is given by the surface potential measurements, as nothing is known about the way the three different polar groups contribute to the surface potential. The hydrocarbon ($C_{34}H_{70}$) derived from phthiocerol melts at 58.5–59.5°, while n-tetratriacontane melts at 72.6–72.8° (15). As mixtures of normal chain higher hydrocarbons have melting points intermediate between those of the components (15), the low melting point of the hydrocarbon cannot be explained on the assumption that it is a mixture of normal chain homologues, and it is therefore probable that it has a branched chain. An x-ray investigation might give some useful information.

α- and β-octadecyl glyceryl ethers, which contain the same polar groups as phthiocerol (two hydroxyls and one ether oxygen) and have a straight chain with 18 carbon atoms, form liquid expanded films with temperatures of half expansion of 31° and 11.5° respectively.[4] As phthiocerol contains 14 or 15 carbon atoms more and the temperature of half expansion in long chain compounds generally increases several degrees for each added carbon atom, the structure of phthiocerol must contain features which counteract the condensing effect of the large number of carbon atoms. Factors that cause expansion (for a given polar group or set of polar groups and a given number of carbon atoms) are (a) branching of the hydrocarbon chain. Apart from possible alkyl side chains, in phthiocerol the methoxyl group very probably plays the rôle of a side chain. (b) The molecule contains a hydrocarbon part that on compression is forced down into the water below the polar groups. This factor is well known from the behavior of long chain esters (17). (c) The polar groups are situated a large distance apart. Long chain dibasic acids and esters give vapor or vapor expanded films (18). In phthiocerol all three of these

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[4] Measurements by N. K. Adam in a paper by Davies *et al.* (16).

factors may contribute to the fact that, in spite of the large number of carbon atoms in the molecule, it forms a liquid expanded monolayer with a temperature of half expansion below room temperature.

We are greatly indebted to Professor R. J. Anderson for the specimen of phthiocerol used, and for his interest in the work. The x-ray experiments on multilayers were carried out in 1939 in the x-ray Crystallography Laboratory, Cambridge, England. We are indebted to Dr. A. J. Bradley for his courtesy. Grants from the Rockefeller Foundation and the Swedish Association against Tuberculosis are gratefully acknowledged.

SUMMARY

Phthiocerol forms on different substrates a typical liquid expanded monolayer. At pH 2, the temperature of half expansion is 17° and the smallest area per molecule to which the monolayers can be compressed before collapse is 30 to 31 sq. Å. In the condensed state the monolayers are solid. The apparent surface moment is 880 millidebyes at the limiting area and falls to 450 at the point of collapse.

It was not found possible to build perfect multilayers of phthiocerol and x-ray experiments on an imperfect Z-deposited film showed that the multilayer was poorly crystalline.

The surface behavior of phthiocerol suggests that it is a very long molecule with only short side chains, and with one or more of the polar groups near one end.

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THE OXIDATION OF ESTRONE BY HYDROGEN PEROXIDE

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One of the outstanding problems in the study of the estrogens is the determination of the metabolism of these compounds. At the present time, little is known about the mechanism of the inactivation process taking place within the body. Dakin (1) has emphasized the similarity between the reactions carried out by the body and those effected through oxidation with H_2O_2. The present investigation of the oxidation of estrone by H_2O_2 was undertaken with the idea that it might indicate a possible metabolic pathway for the inactivation of estrone (Heard and Hoffman (2)).

At the outset of this work, it was expected that H_2O_2 would attack the phenolic portion of the molecule to produce a dihydroxyphenol, since this is a well known reaction of H_2O_2 with phenols (3). However, the evidence has shown that the phenolic ring is not attacked; the oxidation occurs at the carbonyl group with a rupture of Ring D and the production of a hydroxy acid. The free acid rapidly loses the elements of H_2O and is isolated as the lactone (Formula I). The probable explanation for the resistance of the phenolic group to oxidation is that very dilute H_2O_2 and mild conditions were used, whereas the oxidation of monophenols to diphenols usually requires more drastic conditions.
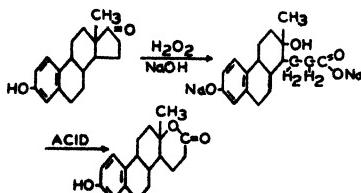
I. Oxidation of estrone by hydrogen peroxide

This reaction is essentially the oxidation of a cyclic ketone to a lactone. The same type of oxidation was obtained with cyclopentanone when the

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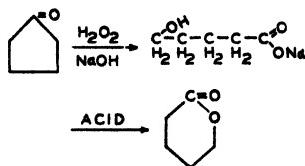
II. Oxidation of cyclopentanone by hydrogen peroxide

latter was treated with H_2O_2 in an alkaline solution (Formula II). This reaction with H_2O_2, therefore, is not confined to estrone, and it may prove to be a general reaction for other cyclic ketones.

EXPERIMENTAL

Oxidation of Estrone with Hydrogen Peroxide—300 mg. of estrone (from mare urine, m.p. 258–260°, $[\alpha]_D = 162.5°$) were dissolved in 60 cc. of 1.0 N NaOH by leaching with small portions of the hot alkali until complete solution was effected.   After cooling to room temperature, the solution was treated with 3.80 cc. of 10 per cent H_2O_2 (Merck's 30 per cent superoxol diluted 1:3 and titrated with standard $KMnO_4$), and the reaction was allowed to proceed at room temperature for 3 days.   The solution was acidified with HCl, and the precipitate was collected by centrifuging or filtering; it was washed with water and dried in a desiccator.   The dry precipitate was leached five to eight times with 25 cc. portions of ethyl ether to remove the unchanged estrone and small amounts of by-products.   The insoluble powder was relatively pure lactone (m.p. > 310°, yield 67 mg.).

The lactone could not be recrystallized from the usual organic solvents because of its extreme insolubility, and the high melting point of the lactone made it impossible to determine its purity by this criterion.   It was, therefore, converted to the acetate, recrystallized, and then hydrolyzed.   Final recrystallization of the lactone was made from dilute pyridine and the crystals so obtained were washed several times with water and 95 per cent alcohol; m.p. 335–340°, softening and browning at 330°.

Analysis—(Sample dried *in vacuo* at 105°)
$C_{18}H_{22}O_3$.   Calculated, C 75.48, H 7.75; found, C 75.45, H 7.66

The above method of oxidation was found to yield the best results even though one-half of the estrone was recovered unchanged and the yield of lactone from the oxidized estrone was only 45 per cent.   Smaller amounts of H_2O_2 gave proportionally a larger amount of unchanged estrone, while greatly increased amounts of H_2O_2 produced larger quantities of highly colored amorphous by-products.   Over-all recoveries indicated some loss of material to the aqueous phase which could not be recovered by extrac-

tion with ether; this loss was marked when excessive amounts of H_2O_2 were used.

When the H_2O_2 is first added to the alkaline solution of estrone, the solution becomes turbid and a fine chalky precipitate settles out. As the reaction progresses and with occasional shaking, the precipitate redissolves. This precipitate is probably unchanged estrone. In one case, it was centrifuged off, washed with water, and recrystallized several times from ethyl alcohol. The crystals gave the typical Kober reaction; m.p. 258–260°, mixed m.p. with estrone 258–260°.

Analysis—(Sample dried *in vacuo* at 105°)
$C_{18}H_{22}O_2$. Calculated, C 79.95, H 8.21; found, C 79.43, H 7.94

The reason for this precipitation is not at all clear. The H_2O_2 contains a small amount of acid, equivalent to an 0.006 N solution, but the addition of an equal amount of 0.006 N HCl does not cause any cloudiness in the solution or precipitation of the estrone. The total amount of estrone initially precipitated by the H_2O_2 is small unless large amounts of H_2O_2 are added.

Properties of Lactone—The high melting point and the relative insolubility of the lactone in all the usual solvents have been noted. The lactone can be dissolved in dilute NaOH by heating and shaking, with the opening of the lactone ring. In concentrated H_2SO_4, it gives a yellow solution with a green fluorescence. The Kober reaction (4) is negative; the initial heating with the reagent produces a yellow color which disappears on dilution with water and subsequent reheating. The Zimmermann test (5) with *m*-dinitrobenzene for the 17-keto group is also negative. The presence of a phenolic hydroxyl is indicated by the orange-yellow color obtained with the diazotized *p*-nitroaniline.

The ultraviolet absorption spectrum of the lactone dissolved in dioxane is identical with the curve obtained for estrone (Fig. 1); maximum absorption at 284 mμ, molecular extinction coefficient = 2200.

The estrogenic activity[1] of the lactone dissolved in dilute aqueous NaOH was tested in spayed mice. The unit necessary for a 50 per cent response was 0.7 γ as compared with the unit for estrone of 0.05 γ. Thus, estrone is 14 times more potent biologically than the lactone.

Derivatives—The lactone formed a very soluble monoacetate that crystallized in characteristic long needles and was very valuable in identifying the lactone.

Acetate—40 mg. of lactone were dissolved in 1 cc. of dry pyridine and 0.5 cc. of acetic anhydride. After standing at room temperature for 24 to 48 hours, the mixture was diluted with ice to 10 cc. and the precipitate

[1] The author is indebted to Dr. S. A. Thayer of St. Louis University for the biological assays.

centrifuged and washed with water. The precipitate was dissolved in 2 cc. of alcohol and crystallized from the hot solution by evaporating and adjusting with water to a concentration of approximately 50 per cent alcohol. Long needles separated on cooling; yield 40 mg., m.p. 143.5–145°.

Analysis—(Sample dried *in vacuo* at 105°)

$C_{20}H_{24}O_4$. Calculated. C 73.13, H 7.37, mol. wt. 328
Found. " 73.08, " 7.21, " " 350

The ultraviolet absorption spectrum of the acetate dissolved in 95 per cent alcohol was similar to that obtained for estrone acetate (Fig. 1). Maximum absorptions of the lactone acetate were at 269 mμ, ϵ = 750, and at 276 mμ, ϵ = 720. The curve for estrone acetate agrees with that previously reported for this substance by John (6). Both estradiol diacetate
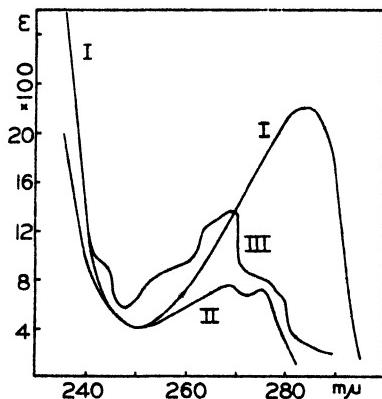
FIG. 1. The ultraviolet absorption spectra of the lactone (Curve I), lactone acetate (Curve II), and estrone acetate (Curve III) plotted as molecular extinction coefficient (ϵ) against wave-length (mμ).

(7) and estriol-3,6,17-triacetate[2] exhibit the two maxima at 269 and 276 mμ.

Hydrolysis of Acetate—35 mg. of the acetate were dissolved in 1.3 cc. of methyl alcohol, and 0.5 cc. of 20 per cent KOH (in methyl alcohol) and 0.2 cc. of water were added. After standing 24 hours at room temperature, the mixture was diluted with water and acidified with HCl. The precipitate was centrifuged off, washed with water, and recrystallized from dilute pyridine; yield 26 mg., m.p. 335°.

The titration of the acetate shows the presence of one acidic group in the molecule in addition to the acetyl radical. 5.515 mg. (0.0168 mM) of the acetate were dissolved in 8 cc. of alcohol, and 2.00 cc. of 0.05 N NaOH were added. After the mixture had stood 48 hours, the neutralization of

[2] Personal communication from Dr. O. Wintersteiner.

excess alkali required 6.34 cc. of 0.01 N HCl. The acetate thus neutralized 0.0366 mM of alkali (theoretical requirement for 2 equivalents, 0.0336 mM).

Semicarbazone—All attempts to form a semicarbazone derivative were unsuccessful. The reaction with the lactone was carried out in pyridine and a reaction with the acetyl derivative was attempted in alcohol. In both cases the unchanged lactone was recovered, as indicated by its high melting point, the absence of nitrogen in the compound, and the identification of the acetate through its melting point and crystal form.

Reduction—The reduction of the lactone acetate was attempted without success, since more than 80 per cent of the starting material was recovered unchanged.

1.0 gm. of sodium was melted under 1 cc. of toluene, and 58 mg. of the lactone acetate in 10 cc. of propyl alcohol were rapidly added under a reflux. After the reaction was completed and the acetate allowed to hydrolyze, the diluted alkaline solution was acidified and extracted with ether. 32 mg. of insoluble lactone were filtered off. The ether-soluble residue, upon acetylation and distillation in a high vacuum, $P = 1 \times 10^{-3}$ mm. of Hg, $t = 100$–$150°$, gave an additional 17 mg. of the lactone acetate.

Methylation—Treatment of the lactone with dimethyl sulfate resulted in a methylation of the phenolic group. The resulting monomethyl ether was stable to both neutral $KMnO_4$ and alcoholic KOH.

46 mg. of the lactone were dissolved in 5 cc. of 10 per cent NaOH and shaken with 1 cc. of dimethyl sulfate. The solution was then heated on a steam bath to decompose the excess reagent, and the methyl ether was precipitated from solution with H_2SO_4. The precipitate was washed with H_2O and recrystallized from dilute methyl alcohol; weight 30 mg., m.p. 166–168°.

Analysis—(Sample dried *in vacuo* at 100°)
$C_{19}H_{24}O_3$. Calculated, OCH_3 10.33; found, OCH_3 10.18

Methylation of the lactone with absolute methyl alcohol and dry HCl yields a non-crystalline mixture of esters in which the lactone ring is opened and the carboxyl group esterified. The liberated hydroxyl group is also replaced by chlorine to the extent of about 30 per cent.

35 mg. of the lactone were refluxed with 8 cc. of absolute methyl alcohol containing 10 per cent dry HCl until solution was effected (6 hours). The solution was poured into ether and washed thoroughly with H_2O. Distillation of the ether and leaching of the residue with small portions of ether left a small residue of unchanged lactone. Since the ether-soluble fraction (35 mg.) could not be crystallized, it was dried and analyzed as such.

Analysis—(Sample dried *in vacuo* at 50°)
Found. OCH_3 9.42, Cl 3.00
Calculated, $C_{19}H_{26}O_4$. " 9.75
" $C_{19}H_{25}O_3Cl$. " 9.10, " 10.54

Methylation with methyl alcohol and concentrated H_2SO_4 as the catalyst was also carried out. Analysis of the oily product from a high vacuum distillation of the ether-soluble residue indicated a mixture of esters in which the carboxyl group was completely methylated and about 50 per cent of the resulting ester dehydrated by loss of the tertiary hydroxyl group.

Analysis—(Sample dried *in vacuo* at 100°)
            Found.   C 73.76, H 8.28, OCH_3 10.34
          Calculated, $C_{19}H_{26}O_4$.   " 71.70, " 8.18,   "   9.75
                " $C_{19}H_{24}O_3$.   " 76.00, " 8.00,   "   10.33

Oxidation of Cyclopentanone—49 gm. of cyclopentanone were dropped into 125 cc. of 20 per cent NaOH, stirred continuously. 100 cc. of 30 per cent H_2O_2 were simultaneously dropped into the solution. The temperature was held at 35–40° by use of a cold water bath. Within several hours, all the cyclopentanone had dissolved and the solution was then extracted with ether to remove any unchanged cyclopentanone. The alkaline solution was acidified with HCl, saturated with NaCl, and the δ-hydroxyvaleric acid was removed by exhaustive extraction with ether. The ether-soluble residue was distilled and the low boiling fraction discarded. 27.5 gm. of the crude δ-valerolactone were recovered from the distillate and purified by means of the barium salt (8).

The distillate was dissolved in a hot $Ba(OH)_2$ solution, and the excess barium was removed with CO_2. After the $BaCO_3$ was filtered off, the solution was taken to dryness *in vacuo*, and the barium salt of δ-hydroxyvaleric acid redissolved in 90 per cent ethyl alcohol. The alcohol was distilled *in vacuo* and the residue taken up in water. A portion of the solution was used for the preparation of the silver salt; the remainder was decomposed with H_2SO_4, extracted with ether, and the ether-soluble residue distilled.

The aqueous solution of the barium salt of δ-hydroxyvaleric acid was precipitated by a solution of $AgNO_3$; the silver salt was filtered, washed with H_2O, and dried in a desiccator.

$C_5H_9O_3Ag$. Calculated, Ag 47.96; found, Ag 48.36

1.1 gm. of silver salt were suspended in H_2O and decomposed with HCl. The filtrate from the AgCl was made alkaline and treated with an excess of $KMnO_4$, at room temperature. The MnO_2 was filtered off and the glutaric acid recovered from the filtrate by acidification and extraction with ether (200 mg.). It was recrystallized several times from benzene; m.p. 94–95°, mixed m.p. with authentic glutaric acid (m.p. 96–97°) 95–97°; neutralization equivalent 68.5, neutralization equivalent for glutaric acid 68.5.

100 mg. of the glutaric acid so obtained plus 0.5 cc. of aniline were heated on an oil bath at 175–190° for 1 hour. After being boiled with 10 cc. of 50 per cent alcohol, the mixture was cooled, filtered, and washed with 50

per cent alcohol.   The dianilide was recrystallized from 95 per cent alcohol;
m.p. 218–219.5°; the dianilide of glutaric acid melts at 221–222°.

In summarizing the evidence for the proposed structure, it is clear that
the oxidation product of estrone contains 3 oxygen atoms.   Only 1 of
these is a hydroxyl group, since it forms a monoacetate and monomethyl
ether.   This hydroxyl must be the original phenolic group, since the
compound gives a positive phenol test with diazotized p-nitroaniline, and
since the absorption spectrum of the lactone and its acetate are so similiar
to the curves for estrone and its acetate that there can be little doubt that
the absorption in both series is due to the same grouping; both curves are
characteristic of phenols.

The other 2 oxygen atoms are present as a lactone, as shown by the
titration of the acidic group, the esterification with methyl alcohol, and the
solubility of the methyl ether in alkali and the lactone in bicarbonate.   All
these reactions indicate the presence of a carboxyl group.   Theoretically,
the compound might be an unsaturated acid instead of a lactone, except
that it is difficult to dissolve in alkali, it is not altered on reduction, and
the methyl ether is stable to permanganate.

In order to obtain a lactone, there must be a break in one of the rings.
The rupture is placed at the carbonyl group because of its disappearance.
There is no doubt of its absence, since the lactone gives a negative Zim-
mermann reaction, it will not form a semicarbazone, and the compound is
not altered by reduction.

The rupture at the carbonyl group gives two possible lactones, depending
upon which side of the carbonyl group the elements of H_2O_2 are added
(Formula III).   In Lactone I, the hydroxyl group is tertiary and the
carboxyl at the end of a chain; in Lactone II, the hydroxyl is primary and
the carboxyl group is linked to a tertiary carbon.   The choice between the
two possible lactones is based on the fact that a carboxyl group linked to a
tertiary carbon atom, as in the second acid, will not esterify with alcohol

III. Possible oxidations of Ring D of estrone by hydrogen peroxide
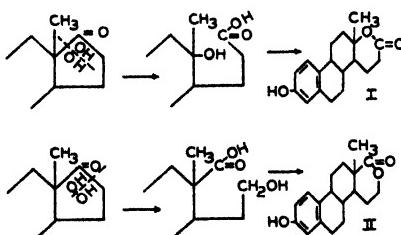
and a catalyst such as HCl or H_2SO_4. The lactone does methylate under these conditions and is, therefore, more compatible with the structure for Lactone I.

This choice is not possible if the methyl group on carbon 13 migrates. However, it is not likely that such a migration would take place under the conditions of the experiment. There is also some indication that the hydroxyl group liberated on opening the lactone ring is actually tertiary.

The basic reaction, *i.e.* the oxidation of a cyclic ketone to a hydroxy acid with subsequent ring closure to a lactone, was confirmed by oxidizing cyclopentanone under similar conditions and identification of the product as δ-valerolactone. Identification was based on analysis of the silver salt of δ-hydroxyvaleric acid and further oxidation of the hydroxy acid to glutaric acid.

SUMMARY

1. The oxidation of estrone in an alkaline solution with H_2O_2 produces a hydroxy acid in which Ring D of the estrone molecule is ruptured at the carbonyl group. The hydroxy acid rapidly loses the elements of H_2O and is isolated as the lactone.

The lactone gives a negative reaction in the Kober and Zimmermann tests, and is one-fourteenth as active in spayed mice as estrone.

2. A similar oxidation of cyclopentanone with H_2O_2 gave δ-valerolactone.

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STUDIES IN SERUM ELECTROLYTES

XIII. ESTIMATION OF TOTAL BASE IN SERUM*

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WITH THE TECHNICAL ASSISTANCE OF DOROTHY BARRUS AND ELEANOR BENDITT

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The measurement of total base of serum (*i.e.*, the total mineral cation-ogens exclusive of NH_4^+ and organic cations) obtained by the usual procedures is time-consuming for routine clinical purposes. For this reason, when information is desired regarding the concentration of electrolytes in serum, a common clinical procedure is either to substitute the concentration of a single individual anion, such as Cl^-, or to add together the equivalent concentrations of Cl^-, HCO_3^-, and Pr^- and to assume an arbitrary correction for the remaining anions. Because of the importance of determining the total electrolyte in the diagnosis and treatment of certain pathological conditions, a method for measuring total base which would be relatively easy of manipulation, rapid, and sparing of material is desirable. To meet such requirements workers on occasion have proposed the use of conductivity measurements.

As early as 1898, Bugarszky and Tangl (1) pointed out that conductivity measurements might be employed as a means of estimating the electrolytes in serum. They observed a diminution in conductivity owing to the presence of serum protein and attempted to correct for this diminution. Palmer, Atchley, and Loeb (2) showed that with NaCl solutions containing either egg albumin or gelatin and adjusted to pH 5.0 and above the conductivity decreased with each increment of protein added; at pH 3.0 the conductivity increased with each increment of protein added. Gram and Cullen (3) found that the conductivity was depressed 2.2 per cent per 1 per cent of serum protein, a value corresponding more closely to that of 2.5 per cent obtained by Bugarszky and Tangl than to the value of 1.5 per cent obtained by Sjöqvist (4). For correcting the observed conductivity of serum, Sunderman, Austin, and Camac (5) observed a linear relationship between the values for corrected conductivity and total base. The present study was directed toward a reinvestigation of this relationship.

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For purposes of relating the concentration of total base in terms of specific conductance and concentrations of protein, measurements for total base, conductivity, and specific gravity were made in forty-seven specimens of serum from patients suffering from miscellaneous pathological conditions.

The conductivity measurements were made by means of a modified sugar ash bridge assembly[1] and a pipette type of conductivity cell[2] of 1.5 ml. capacity which have been described (6). To avoid polarization effects, cells with constants between 8 and 10 reciprocal cm. were employed. The high constants in the cells were obtained by constricting the lumen of the tube between the electrodes. The specific gravity measurements were made by weighing serum in specific gravity bottles of 2 ml. capacity and the concentration of protein was calculated from these measurements by the method of Moore and Van Slyke (7). Since chemical analyses for total base are likely to involve a greater error than the relatively simple measurements of conductivity and specific gravity, the total base analyses by the procedure of Hald (8) were made in sets of six, two samples of a known salt mixture, two samples of a given serum, and two samples of the same serum to which had been added a known quantity of the salt mixture. In general, when variations amounting to more than 2 per cent appeared, these analyses were excluded from the data.

The data obtained in the forty-seven specimens of serum in our series are given in Table I. From these data a formula was sought for deriving the concentration of total base from measurements of the specific gravity (or protein) and the specific conductance.

Derivation of Equations—L = specific conductance $\times 10^3$ at 25°; L_c = corrected conductivity $\times 10^3$ at 25°; L_o = observed conductivity $\times 10^3$ at 25°; d = factor; k = constant; Pr = protein, gm. per 100 ml.; B = total base, milliequivalents per liter; μ = ionic strength; G = (specific gravity 20°/20° $-$ 1.0000) 10^4. The value of G expressed in this manner permits the last three figures of specific gravity to be used as whole numbers.

Equation 1—The studies of Palmer, Atchley, and Loeb indicated that Equation 1 might be employed to obtain corrected conductivity values for

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[1] Made for us by the Leeds and Northrup Company, Philadelphia.

[2] The pipette conductivity cells have been made by Mr. James D. Graham, glassblower of the University of Pennsylvania. Electrodes according to the following specifications have been found satisfactory: disk-shaped platinum plates 0.50 cm. in diameter, 0.038 cm. in thickness, with platinum wire 0.054 cm. in diameter and 1.2 cm. in length attached along the entire diameter of the plates. The internal diameter of the cells is 4.5 mm.; the distance between the electrodes, approximately 17 mm. The surfaces of the electrodes are coated with platinum black.

their saline solutions of albumin and gelatin adjusted to pH 5.0 and 8.1, since the observed conductivity in their solutions was decreased a uniform amount for each additional increment of protein.

(1) $$L_c = L_o + d_1 \text{Pr}$$

Evaluation of factor d_1 may be made if it be assumed that over the range of concentration of total electrolytes observed clinically (*i.e.* 100 to 200 milliequivalents per liter) the relation between base and specific conduct-

TABLE I

Measurements Obtained on Samples of Human Serum

Subgroup A								Subgroup B			
Sample No.	Observed total base	Observed specific conductance, 25°	Sp. gr., 20°/20°	Sample No.	Observed total base	Observed specific conductance, 25°	Sp. gr., 20°/20°	Sample No.	Observed total base	Observed specific conductance, 25°	Sp. gr., 20°/20°
	m. eq. per l.	*mhos × 10³*			*m. eq. per l.*	*mhos × 10³*			*m. eq. per l.*	*mhos × 10³*	
1	146.2	12.03	1.0257	29	138.4	11.35	1.0238	2	147.7	11.86	1.0262
3	143.0	11.78	1.0253	31	150.5	12.46	1.0240	4	146.6	11.86	1.0269
7	145.0	11.69	1.0291	32	145.0	12.21	1.0208	6	131 0	10.32	1.0276
12	147.2	11.86	1.0272	33	142.8	11.91	1.0230	8	163.0	13.50	1.0320
16	137.7	10.83	1.0312	34	138.5	11.86	1.0218	9	146.0	11.86	1.0276
17	149.5	11.95	1.0247	35	135.5	10.57	1.0268	10	148 5	11.91	1.0266
18	144.1	12.03	1.0194	36	146.5	12.29	1.0257	11	143.7	12.21	1.0214
19	145.4	11.95	1.0233	37	139.0	11.22	1.0204	13	138.1	12.21	1.0212
21	152.6	12.03	1.0260	38	143.0	11.78	1.0228	15	150 2	12 94	1.0191
22	145.0	11.78	1.0230	39	141.2	11.60	1.0227	20	144.6	11.78	1.0252
23	146.4	12.03	1.0240	42	146.0	11.86	1.0275	25	135 3	11.69	1.0215
24	141.6	11.86	1.0239	43	142.8	11.73	1.0265	30	151.0	12.29	1.0233
26	149.7	12.16	1.0281	44	144.0	11.82	1.0264	41	134.4	10.49	1.0234
27	146.4	11.82	1.0252	46	137.0	11.91	1.0241	45	145.0	12.12	1.0223
28	137.7	11.35	1.0296	47	143.0	12.03	1.0199	48	147.7	12.03	1.0231
								49	153.0	12.55	1.0246
								50	142.1	11.52	1.0283

ance, in the absence of protein effect, is approximately the same as that between NaCl and its specific conductance in these concentrations. This relation, derived from data contained in the International Critical Tables, may be expressed as follows (9):

(1, *a*) $$B = 10.37L - 10.65$$

Since in Equation 1 $L_c = L$, by substitution, Equation 1 becomes

(1, *b*) $$B = 10.37 (L_o + d_1 \text{Pr}) - 10.65$$

The average value of d_1 calculated from the data given in Table I is 0.517. When this value is substituted,

(1, c) $B = 10.37L_o + 5.36Pr - 10.65$

Equation 2—A statistical calculation of the three variates (specific conductance, specific gravity, and total base) observed in the forty-seven specimens of Table I was made by means of partial regression (10) and the constants evaluated. This calculation yielded Equation 2, similar in type to Equation 1, *c*,

(2) $B = 8.62L_o + 0.06G + 27.26$

When the equation of Moore and Van Slyke (7) relating the specific gravity to serum protein is substituted, Equation 2 may be expressed,

(2, a) $B = 8.62L_o + 1.75Pr + 31.41$

Equation 3—Bugarszky and Tangl and Gram and Cullen suggested the use of Equation 3 for correcting conductivity, since it appeared to fit their data better than Equation 1. Equation 3 is based upon the assumption that the observed conductivity is decreased percentilely owing to the protein concentration.

(3) $$L_c = \frac{100L_o}{100 - d_2\,Pr}$$

If Equation 1, *a* is substituted, Equation 3 becomes

(3, a) $$B = \frac{1037L_o}{100 - d_2\,Pr} - 10.65$$

The average value of d_2 calculated from the data given in Table I is 3.46. When this value is substituted,

(3, b) $$B = \frac{1037L_o}{100 - 3.46Pr} - 10.65$$

Equation 4—It is known that the mobility of an ion is decreased when the ion concentration is increased and an equation for this effect has been offered by Onsager (11). The constants in this equation involve the effects of viscosity, dielectric constant, temperature, and charge of ion. By making the approximation that the change in dielectric constant is negligible over the range of our observations (doubtful) and that the viscosity is a function of the protein present, an empirically simplified form of the Onsager equation may be derived and expressed as follows:

(4) $$L_c = L_o + \frac{\mu\sqrt{\mu k}}{Pr} + \mu\sqrt{\mu k_1}$$

If the ionic strength is regarded as essentially a constant for the limited range of observed values in our series, Equation 4 becomes

$$(4,\,a) \qquad\qquad L_c = L_o + \frac{k_2}{\mathrm{Pr}} + k_3$$

Substituting values of B for L_c, we have

$$(4,\,b) \qquad\qquad B = k_4 L_o + \frac{k_5}{\mathrm{Pr}} + k_6$$

By means of the data contained in Table I, the constants in Equation 4, b may then be derived statistically by means of partial regression (10). When these evaluated constants are substituted, Equation 4, b becomes

$$(4,\,c) \qquad\qquad B = 10.19 L_o - \frac{69.45}{\mathrm{Pr}} + 35.16$$

Comparison of Equations—In order to test and compare the values of total base calculated by means of the four different types of equation, the

<div align="center">

TABLE II

Comparison of Calculated and Observed Total Base

</div>

	Data from entire group (47 observations); constants of equations given in text		Data from Subgroup B (17 observations); constants of equations calculated from Subgroup A (30 observations)		
Equation No.	Mean percentile deviation	Range of percentile deviation	Standard percentile deviation	Mean percentile deviation	Range of percentile deviation
1, b	±2.5	+8.1 to −6.2	2.89	±2.3	+6.7 to −5.4
2	±1.5	+3.9 " −5.4	2.10	±1.6	+5.1 " −2.5
3, a	±3.3	+15.7 " −8.1	4.56	±3.3	+13.4 " −7.5
4, b	±1.5	+5.7 " −3.6	2.15	±1.6	+5.4 " −2.3

data given in Table I were divided by random sampling into two separate subgroups of thirty and seventeen specimens respectively. The constants for each type of equation were then evaluated from the data of Subgroup A (thirty samples, Table I) and the equations thus obtained were used to calculate the values of total base for Subgroup B (seventeen samples, Table I).

In Table II are given the mean percentile deviation and the range of the percentile deviation for each of the four equations derived from the entire data. In addition, the standard percentile deviation, the mean percentile deviation, and the range of the percentile deviation are given for each of the four equations with constants calculated from the data of Subgroup A and applied to those of Subgroup B. It will be seen that calculations derived from Equations 2 and 4, b yield the best values and compare with the

observed values in forty-seven determinations with an average deviation of ±1.5 per cent, respectively.

DISCUSSION

For routine clinical purposes we use either Equation 2 or Equation 4, *c* for the calculation of total base. It might be pointed out, however, that the form of Equation 4, *c* is in accord with the experimental observations of diminishing conductivity with increasing concentrations of protein.

Gram and Cullen showed that the presence of glucose and urea even in the concentrations observed in severe pathological conditions produced negligible diminution in conductivity. In several of the specimens in our series the concentration of such non-electrolytes as cholesterol, urea, and glucose was elevated. In these specimens the agreement of calculated with the analyzed values appeared not to be significantly affected.

In the modified sugar ash bridge which we employ, a compensating resistance may be inserted to obtain direct readings of specific conductance at 25°. The use of the compensator has proved to be time-saving and convenient.

It might be emphasized that there need be essentially no loss of serum for measuring either the conductivity or specific gravity. After these measurements are made, the serum may be utilized for other types of analyses.

SUMMARY

A method is given by which the concentration of total base of serum may be calculated from measurements of specific conductance and either specific gravity or serum protein. The method is simple and economical of both time and material, so that it would seem to afford a method of choice for clinical studies.

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THE SYNTHESIS OF D-ERYTHRO- AND D-THREO-α-AMINO-β,γ-DIHYDROXY-n-BUTYRIC ACIDS*

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In 1931 Klenk and Diebold (1) reported that sphingosine, upon oxidative degradation, gave rise to myristic acid and an optically active dihydroxyaminobutyric acid, $[\alpha]_D^{13} = -33.4°$, which these authors characterized as an α-amino-β,γ-dihydroxy-n-butyric acid. 5 years later Fischer and Feldmann (2), starting from D-glyceric aldehyde, synthesized an optically active α-amino-β,γ-dihydroxy-n-butyric acid, $[\alpha]_D^{20} = -13.7°$, and, in order to explain the difference in the specific rotation of their amino acid and that of Klenk and Diebold, suggested that the amino acid with $[\alpha]_D^{20} = -13.7°$ was a mixture of the two expected disastereoisomers, one of which was identical with, or an antipode of, the amino acid obtained from sphingosine.

We repeated the synthesis of Fischer and Feldmann and obtained, as they did, the amino acid with $[\alpha]_D^{24} = -13.7°$ in good yield. However, in addition another α-amino-β,γ-dihydroxy-n-butyric acid, with $[\alpha]_D^{24} = 16.0°$, was isolated from the reaction mixture. It is the purpose of this communication to show that the amino acid with $[\alpha]_D^{24} = -13.7°$ is not a mixture of disastereoisomers, but is D-threo-α-amino-β,γ-dihydroxy-n-butyric acid, and that the amino acid with $[\alpha]_D^{24} = 16.0°$ is D-erythro-α-amino-β,γ-dihydroxy-n-butyric acid.

An α-benzamido-β,γ-dihydroxy-n-butyrolactone, m.p. 210–211°, was obtained when the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$ was benzoylated. This lactone when treated with phenylhydrazine gave an α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide, $[\alpha]_D^{24} = -15.9°$. The partial hydrolysis of this phenylhydrazide led to the regeneration of the α-benzamido-β,γ-dihydroxy-n-butyrolactone, m.p. 210–211°, and when this lactone was hydrolyzed the original α-amino-

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* The prefixes erythro and threo define the relative configuration about the 2 asymmetric carbon atoms bearing the amino and hydroxyl groups; the letters D and L relate the configuration about the asymmetric carbon atom bearing the hydroxyl group with the configuration about the asymmetric carbon atom present in D- or L-glyceric aldehyde.

β,γ-dihydroxy-n-butyric acid was recovered. If the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$ were a mixture of diastereoisomers, one would expect that the above transformations would bring about at least a partial resolution, and a concomitant change in specific rotation. As this did not occur, we concluded that the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$ is not a mixture but is one of the two expected disastereoisomers. The correctness of this conclusion was demonstrated when the same series of reactions was applied to the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = 16.0°$. In this instance benzoylation of the amino acid led to the formation of an α-benzamido-β,γ-dihydroxy-n-butyric acid with a melting point of 135–136°. Thus we have a situation in which benzoylation of one diastereoisomer results in the formation of a stable benzamido lactone and benzoylation of the other diastereoisomer results in the formation of a stable benzamido acid.[1] Since the physical and chemical properties of the benzamido acid and the benzamido lactone are so different, it is clear that any diastereoisomeric impurity in one or the other amino acid would not survive the benzoylation reaction and since the two benzoyl derivatives could be converted into the amino acids from which they were formed without altering the original specific rotations of the amino acids it is obvious that the latter compounds are pure diastereoisomers.[2]

Bergmann and coworkers (3) have shown that the enzymatic synthesis of the amides and phenylhydrazides of acylated amino acids by purified papain proceeds only when the acylated amino acid has the L configuration around the carbon atom bearing the potential amino group. The incubation of sodium α-benzamido-β,γ-dihydroxy-n-butyrate, $[\alpha]_D^{24} = 31.3°$, prepared from the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$ with phenylhydrazine, purified papain, and cysteine hydrochloride led to the formation of an α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide which was identical with the phenylhydrazide prepared by condensing phenylhydrazine with the α-benzamido-β,γ-dihydroxy-n-butyro-

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[1] Scale models of the two diastereoisomeric benzamido lactones revealed that in the case of the erythro compound some of the possible positions, produced by rotation around carbon-nitrogen and carbon-oxygen single bonds, resulted in absurd interatomic distances. This was not the case with the threo compound and considering the possibility of restricted rotation around the various single bonds it is understandable why one diastereoisomer should form a stable benzamido acid and the other a stable benzamido lactone. It is clear that these considerations also lead to the assignment of the erythro configuration to the amino acid $[\alpha]_D^{24} = 16.0°$ and the threo configuration to the amino acid $[\alpha]_D^{24} = -13.7°$.

[2] The benzamido acid, derived from the α-amino-β,γ-dihydroxy-n-butyric acid, $[\alpha]_D^{24} = 16.0°$, was also converted into the corresponding phenylhydrazide, $[\alpha]_D^{25} = 87.8°$, from which the original amino acid, $[\alpha]_D^{24} = 15.7°$, was obtained by hydrolysis.

lactone derived from the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$. Furthermore the enzymatically synthesized phenylhydrazide was hydrolyzed stepwise to give the α-amino-β,γ-dihydroxy-n-butyrolactone with a melting point of 210–211°, and the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$. These experiments not only offer another demonstration of the homogeneity of the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = -13.7°$ but also provide substantial evidence that this amino acid is the D-threo-α-amino-β,γ-dihydroxy-n-butyric acid.

Lutz and Jirgensons (4) have shown that the specific rotation of an amino acid in aqueous solution is dependent, within limits, upon the pH of the solution and that with increasing acid concentration the specific rotation changes in a positive sense for L antipodes and in a negative sense for D antipodes. When these principles were applied to the two diastereoisomeric α-amino-β,γ-dihydroxy-n-butyric acids, it was found that the specific rotation of the amino acid with $[\alpha]_D^{24} = -13.7°$ changed in a positive sense and the specific rotation of the amino acid with $[\alpha]_D^{24} = 16.0°$ changed in a negative sense, with increasing acid concentration. Thus we have additional evidence consistent with the interpretation that the amino acid with $[\alpha]_D^{24} = -13.7°$ is D-threo-α-amino-β,γ-dihydroxy-n-butyric acid and the one with $[\alpha]_D^{24} = 16.0°$ is D-erythro-α-amino-β,γ-dihydroxy-n-butyric acid.

The results of the investigations of Krebs (5) on D-amino acid oxidase were utilized in a study of the action of this enzyme upon the two disastereoisomeric α-amino-β,γ-dihydroxy-n-butyric acids. As the rate of oxygen consumption, in c.mm. per hour at 30°, for the α-amino-β,γ-dihydroxy-n-butyric acid with $[\alpha]_D^{24} = 16.0°$ was 27.6 and that for the acid with $[\alpha]_D^{24} = -13.7°$ was 0.0, we can conclude that this experiment provides still further evidence that the amino acid with $[\alpha]_D^{24} = 16.0°$ is D-erythro-α-amino-β,γ-dihydroxy-n-butyric acid and that the other acid is D-threo-α-amino-β,γ-dihydroxy-n-butyric acid.

D-Erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid was refluxed with anhydrous butanol to give a reaction product which was composed of 2 parts of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyrolactone and 1 part of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone. The mixture was dissolved in aqueous alkali and acidification of this solution led to the isolation of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid and D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone. This conversion of one diastereoisomer into the other is still further proof of the correctness of our conclusions regarding the homogeneity and configuration of the two diastereoisomeric D-α-amino-β,γ-dihydroxy-n-butyric acids.

The synthesis and characterization of the two theoretically possible

diastereoisomeric D-α-amino-β,γ-dihydroxy-n-butyric acids have made it possible to compare the properties of these structurally unambiguous amino acids, or those of their antipodes, with those exhibited by the aminodihydroxy-n-butyric acid which Klenk and Diebold (1) obtained from sphingosine. As the specific rotation of this latter amino acid differs markedly from those of the known α-amino-β,γ-dihydroxy-n-butyric acids,[3] we conclude that the amino acid obtained by Klenk and Diebold (1) cannot be an α-amino-β,γ-dihydroxy-n-butyric acid, if the specific rotation that they report is correct. On the basis of our present knowledge we must therefore reject the structural formula which Klenk and Diebold (1) proposed for sphingosine and proceed to consider other possible structures. Such investigations are now in progress in this laboratory.

EXPERIMENTAL[4]

D-Threo-α-amino-β,γ-dihydroxy-n-butyric Acid—To a solution of 200 gm. of 1,2,5,6-diacetone mannitol, m.p. 120–121°, in 2 liters of anhydrous ethyl acetate, were added, in ten portions, with vigorous stirring at 25°, 337.6 gm. of lead tetraacetate. The precipitated lead salts were discarded, and the filtrate freed of solvent by distillation at atmospheric pressure. The residue was dissolved in 450 ml. of methanol and 41 gm. of anhydrous hydrogen cyanide added to the chilled solution, contained in a pressure bottle. The reaction mixture was maintained at 37° for 2 days, saturated, at 0°, with anhydrous ammonia, and again allowed to stand at 37° for 2 days. The excess ammonia was removed by evaporation *in vacuo* at 30°, the residue dissolved in 700 ml. of methanol, and 1600 ml. of concentrated hydrochloric acid added to the chilled solution. After standing at 37° for 2 days the solution was saturated, at 0°, with hydrogen chloride and allowed to stand at 37° for 1 day. The precipitated ammonium chloride was discarded and the filtrate freed of excess hydrogen chloride by repeated evaporation *in vacuo* at 25°. The residue was dissolved in 2.5 liters of water and sufficient crystalline barium hydroxide added to allow the complete removal of ammonia upon subsequent evaporation of the solution. The filtered ammonia-free solution was successively treated with lead carbonate, silver carbonate, hydrogen sulfide, and norit, and the colorless solution evaporated to dryness *in vacuo* at 35°. The residue was dissolved in 300 ml. of hot water, and after the addition of 400 ml. of methanol, the solution was allowed to stand for 16 hours at 5°. The crystalline product was collected, dried, and recrystallized first from 70 per cent aqueous

[3] As the specific rotation of the known α-amino-β,γ-dihydroxy-n-butyric acids is practically independent of the temperature, a direct comparison of specific rotations is permissible.

[4] Microanalyses by Dr. G. Oppenheimer and Mr. G. A. Swinehart.

methanol and then from water to give 41.5 gm. of D-threo-α-amino-β,γ-dihydroxy-n-butyric acid, hexagonal platelets, m.p. 215°, with decomposition.

Analysis—$C_4H_9O_4N$ (135.1). Calculated. C 35.6, H 6.7, N 10.4
Found. " 35.4, " 6.7, " 10.4
Specific Rotation—$[\alpha]_D^{24} = \dfrac{-0.62° \times 2}{1 \times 0.0906} = -13.7°$ (in water)

D-Erythro-α-amino-β,γ-dihydroxy-n-butyric Acid—Methanol (300 ml.) was added to the mother liquor remaining after the separation of the D-threo-α-amino-β,γ-dihydroxy-n-butyric acid and after standing at 5° for 2 days the oil, which had separated on the addition of the methanol, solidified. The solid was collected and recrystallized twice from 60 per cent aqueous methanol, and then from water, to give 7.6 gm. of a crystalline product, m.p. 188–190°, with decomposition, $[\alpha]_D^{24} = 6.8°$. As microscopic examination of this product revealed the presence of two types of crystals, hexagonal platelets and short thick needles, it was subjected to three recrystallizations from 15 parts of 45 per cent aqueous methanol and one recrystallization from water, to give 3.1 gm. of D-erythro-α-amino-β,γ-dihydroxy-n-butyric acid, short thick needles, m.p. 192–194°, with decomposition.

Analysis—$C_4H_9O_4N$ (135.1). Calculated. C 35.6, H 6.7, N 10.4
Found. " 35.6, " 6.7, " 10.4
Specific Rotation—$[\alpha]_D^{24} = \dfrac{0.44° \times 2}{1 \times 0.055} = +16.0°$ (in water)

D-Threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone—Benzoyl chloride (28 gm.) and 230 ml. of 2 N sodium hydroxide were added, at 5°, in ten equal portions at 30 minute intervals, to 6.76 gm. of D-threo-α-amino-β,γ-dihydroxy-n-butyric acid, $[\alpha]_D^{24} = -13.7°$, dissolved in 12.5 ml. of water and 37.5 ml. of 2 N sodium hydroxide (6). The reaction mixture was acidified, filtered, the filtrate extracted with ether, the aqueous phase concentrated *in vacuo* to 100 ml., and the concentrate allowed to stand at 5° for 5 days. The solid was collected, and recrystallized from 50 per cent aqueous ethanol to give 4.7 gm.[5] of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone, needles, m.p. 210–211°, with decomposition.

Analysis—$C_{11}H_{11}O_4N$ (221.1). Calculated. C 59.7, H 5.0, N 6.3
Found. " 59.9, " 5.1, " 6.3
Specific Rotation—The lactone, 37.7 mg., was dissolved in 1.92 ml. of 0.0876 N sodium hydroxide and the solution made up to 2.0 ml.; $[\alpha]_D^{24} = \dfrac{0.59° \times 2}{1 \times 0.0377} = +31.3°$

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[5] An additional 1.1 gm. were obtained on working up the mother liquors.

D-Threo-α-benzamido-β,γ-dihydroxy-*n*-butyrolactone, m.p. 210–211°, (250 mg.) was refluxed for 4 hours with 5 ml. of 8 per cent hydrochloric acid, the hydrolysate extracted with ether, the aqueous phase freed of chloride ion, and evaporated to dryness. 1.5 ml. of methanol were added to the residue dissolved in 1 ml. of water, and after standing for 16 hours at 5° the precipitate was collected and dried to give 84.2 mg. (55 per cent) of *D*-threo-α-amino-β,γ-dihydroxy-*n*-butyric acid, hexagonal platelets, m.p. 215°, with decomposition.

$$\text{Specific Rotation—}[\alpha]_D^{24} = \frac{-0.56° \times 2}{1 \times 0.084} = -13.4° \text{ (in water)}$$

D-*Erythro*-α-*benzamido*-β,γ-*dihydroxy*-*n*-*butyric*   *Acid*—*D*-Erythro-α-amino-β,γ-dihydroxy-*n*-butyric acid, $[\alpha]_D^{24} = 16.0°$, was benzoylated in a manner similar to that described above and from 0.8 gm. of the amino acid we obtained 1.04 gm. of *D*-erythro-α-benzamido-β,γ-dihydroxy-*n*-butyric acid, needles, m.p. 135–136°, after recrystallization from water.[6]

Analysis—$C_{11}H_{13}O_5N$ (239.1).   Calculated.   C 55.2, H 5.4, N 5.9
                                 Found.   " 55.4, " 5.8, " 5.8
Specific Rotation—The acid, 37.7 mg., was dissolved in 1.80 ml. of 0.0876 N sodium hydroxide and the solution made up to 2.0 ml.; $[\alpha]_D^{24} = \dfrac{-0.44° \times 2}{1 \times 0.0377} = -23.3°$

A solution of 1 gm. of *D*-erythro-α-benzamido-β,γ-dihydroxy-*n*-butyric acid in 25 ml. of anhydrous butanol was refluxed for 3 hours. The solvent was removed and the residue recrystallized from 7 ml. of absolute ethanol to give 0.6 gm. of lactone, platelets, m.p. 135–138°.

Analysis—$C_{11}H_{11}O_4N$ (221.1).   Calculated.   C 59.7, H 5.0, N 6.3
                                 Found.   " 59.8, " 5.4, " 6.5
Specific Rotation—The lactone, 37.8 mg., was dissolved in an equivalent amount of 0.0876 N sodium hydroxide and the solution made up to 2.0 ml.; $[\alpha]_D^{24} = \dfrac{-0.11° \times 2}{1 \times 0.0378}$ = $-5.8°$

A mixture of 2 parts of *D*-erythro-α-benzamido-β,γ-dihydroxy-*n*-butyrolactone and 1 part of *D*-threo-α-benzamido-β,γ-dihydroxy-*n*-butyrolactone, when converted into the sodium salts of the corresponding acids, would be expected to have a specific rotation of $[\alpha]_D^{24} = -5.8°$. The presence of the two diastereoisomers in the above product was demonstrated as follows: 200 mg. of the lactone, $[\alpha]_D^{24} = -5.8°$, were dissolved in 2 ml. of N sodium

[6] *D*-Erythro-α-benzamido-β,γ-dihydroxy-*n*-butyric acid is readily soluble in cold aqueous potassium bicarbonate. As *D*-threo-α-benzamido-β,γ-dihydroxy-*n*-butyrolactone is insoluble in this reagent, it is obvious that we have here a second and more elegant method for separating the two diastereoisomers.

hydroxide and the solution cautiously acidified, at 0°, with 12 N hydrochloric acid. The precipitate that had formed was collected to give 142 mg. of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid, m.p. 135–136°, and was soluble in cold aqueous potassium bicarbonate. The filtrate (above) was kept at 5° for 1 day, and the crystalline precipitate collected, and recrystallized from water, to give 20 mg. of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone, m.p. 210–211°, insoluble in cold aqueous potassium bicarbonate.

D-Erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid (500 mg.) was refluxed for 5 hours with 10 ml. of 8 per cent hydrochloric acid, the hydrolysate extracted with ether, and the aqueous phase freed of chloride ion and evaporated to dryness. 7 ml. of methanol were added to a hot filtered solution of the residue in 2 ml. of water, and after standing overnight at 0°, the precipitate was collected, dried, and recrystallized from 7 ml. of 70 per cent methanol, to give 42 mg. of D-erythro-α-amino-β,γ-dihydroxy-n-butyric acid, needles, m.p. 192–194°, with decomposition.

$$\text{Specific Rotation—}[\alpha]_D^{25} = \frac{0.33° \times 2}{1 \times 0.042} = +15.7° \text{ (in water)}$$

D-Threo-α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide — A mixture of 200 mg. of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone, m.p. 210–211°, and 1.5 ml. of phenylhydrazine was heated, at 100° in an atmosphere of nitrogen, for 3 hours. 5 ml. of ether were added to the clear solution, and the latter allowed to stand, at 5°, for 16 hours. The crystalline precipitate was collected and recrystallized from 95 per cent ethanol to give 168 mg. (57 per cent) of the phenylhydrazide of D-threo-α-benzamido-β,γ-dihydroxy-n-butyric acid, platelets, m.p. 169°.

Analysis—$C_{17}H_{19}O_4N_3$ (329.2). Calculated. C 61.9, H 5.9, N 12.8
Found. " 61.9, " 5.9, " 13.0

$$\text{Specific Rotation—}[\alpha]_D^{24} = \frac{-0.48° \times 2}{1 \times 0.0604} = -15.9° \text{ (in pyridine)}; [\alpha]_D^{24} = \frac{-0.14° \times 2}{1 \times 0.0308} =$$
−9.1° (in glacial acetic acid)

In order to provide evidence regarding the configuration around the asymmetric carbon atom bearing the benzamido group the phenylhydrazide was synthesized enzymatically according to the procedure of Bergmann and coworkers (3). 4.10 gm. of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone were dissolved in the minimum amount of N sodium hydroxide, and the resulting solution adjusted to pH 4.7 with glacial acetic acid. After the addition of 15 ml. of citrate buffer (pH 5.0), 20 ml. of papain-buffer solution,[7] 1.85 ml. of phenylhydrazine, and 0.2 gm. of

[7] Prepared by dissolving 180 mg. of purified papain (3) in 10 ml. of water and 10 ml. of citrate buffer (pH 5.0).

cysteine hydrochloride to the above solution, it was adjusted to pH 4.7, made up to 100 ml., and incubated at 40° for 1 week. After the solution was cooled to 0°, the phenylhydrazide was collected and recrystallized from 95 per cent ethanol to give 2.96 gm. (49 per cent) of D-threo-α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide, platelets, m.p. 169°, mixed melting point with the non-enzymatically prepared phenylhydrazide 169°.

$$\textit{Specific Rotation---}[\alpha]_D^{25} = \frac{-0.48° \times 2}{1 \times 0.0603} = -15.9° \text{ (in pyridine)}$$

In accordance with a reaction observed by Hann and Hudson (7) 2.3 gm. of D-threo-α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide, m.p. 169°, were refluxed for 2 hours with 1.76 gm. of cupric sulfate pentahydrate dissolved in 23 ml. of water. The cuprous oxide was removed and the filtrate placed in the cold room overnight. The solid was collected and recrystallized from 50 per cent ethanol to give 0.5 gm. of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone, needles, m.p. 210–211°, with decomposition.

Analysis—$C_{11}H_{11}O_4N$ (221.1). Calculated. C 59.7, H 5.0, N 6.3
                                   Found.   " 59.4, " 5.1, " 6.3

$$\textit{Specific Rotation---}[\alpha]_D^{25} = \frac{0.60° \times 2}{1 \times 0.0377} = +31.8° \text{ (in an equivalent amount of}$$
sodium hydroxide)

The filtrate remaining after the separation of D-threo-α-benzamido-β,γ-dihydroxy-n-butyrolactone was freed of cupric and sulfate ions and evaporated to dryness. The residue dissolved in 8 ml. of 8 per cent hydrochloric acid was refluxed for 5 hours and upon working up the hydrolysate we obtained 224 mg. of D-threo-α-amino-β,γ-dihydroxy-n-butyric acid, hexagonal platelets, m.p. 215°, with decomposition.

$$\textit{Specific Rotation---}[\alpha]_D^{25} = \frac{-0.66° \times 2}{1 \times 0.100} = -13.2° \text{ (in water)}$$

D-Erythro-α-benzamido-β,γ-dihydroxy-n-butyr-(N-phenyl)hydrazide — A mixture of 100 mg. of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid, m.p. 135–136°, and 2 ml. of phenylhydrazine was heated, at 100° in an atmosphere of nitrogen, for 5 hours, 10 ml. of ether were added to the chilled solution, and the latter allowed to stand in an ice bath for 2 hours. The precipitate was collected, washed with ether, and recrystallized from 95 per cent ethanol to give 84 mg. (57 per cent) of the phenylhydrazide of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid, needles, m.p. 203–204°, with decomposition.

Analysis—$C_{17}H_{19}O_4N_3$ (329.2).   Calculated.   C 61.9, H 5.9, N 12.8
                                    Found.   " 61.9, " 5.9, " 12.8

Specific Rotation—$[\alpha]_D^{23} = \dfrac{0.72° \times 2}{1 \times 0.0164} = +87.8°$ (in pyridine)

A mixture of 500 mg. of the phenylhydrazide of D-erythro-α-benzamido-β,γ-dihydroxy-n-butyric acid, m.p. 203–204°, and 5 ml. of 8 per cent hydrochloric acid was refluxed for 3 hours, the hydrolysate extracted with ether, and the aqueous phase freed of chloride ion and evaporated to dryness. The residue was dissolved in 1 ml. of hot water, 3 ml. of methanol added, and the solution placed in the cold room overnight. The solid was collected and recrystallized from 45 per cent methanol to give 49 mg. of D-erythro-

TABLE I

Relation between Acidity and Specific Rotation of Aqueous Solutions of
D-Threo-α-amino-β,γ-dihydroxy-n-butyric Acid

$\dfrac{\text{M hydrochloric acid}}{\text{M amino acid}}$	$[\alpha]_D^{23}$
	degrees
0.0	−13.7
0.50	−8.7
1.00	−3.5
1.50	−2.8
1.75	−2.4
2.00	−1.8
3.00	−1.8
4.80	−1.7

α-amino-β,γ-dihydroxy-n-butyric acid, short thick needles, m.p. 192–194°, with decomposition.

Specific Rotation—$[\alpha]_D^{23} = \dfrac{0.36° \times 2}{1 \times 0.049} = +14.7°$ (in water)[8]

Relation between Acidity and Specific Rotation of Aqueous Solutions of D-Threo- and D-Erythro-α-amino-β,γ-dihydroxy-n-butyric Acid—In all experiments relating to D-threo-α-amino-β,γ-dihydroxy-n-butyric acid, 270 mg. of the amino acid, $[\alpha]_D^{24} = -13.7°$, were dissolved in varying amounts of N hydrochloric acid and the solutions made up to 10 ml. (Table I).

In all experiments relating to D-erythro-α-amino-β,γ-dihydroxy-n-

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[8] The low rotation observed in this case is probably due to the presence of decomposition products produced by the action of hydrochloric acid on the amino acid. Because of the small amount of material available, no attempt was made to raise the rotation by further recrystallization.

TABLE II

Relation between Acidity and Specific Rotation of Aqueous Solutions of
D-Erythro-α-amino-β,γ-dihydroxy-n-butyric Acid

M hydrochloric acid / M amino acid	$[\alpha]_D^{23}$
	degrees
0.0	16.0
1.0	−11.1
2.0	−18.5
3.0	−23.0

TABLE III

Oxygen Uptake at 30°

Substrate	Rate of oxygen consumption
	c.mm. per hr.
DL-Alanine	237.0
DL-Serine..	145.0
D-Erythro acid, $[\alpha]_D = 16.0°$..	27.6
D-Threo acid, $[\alpha]_D = -13.7°$.	0.0
Control.......	0.0

TABLE IV

Enzymatic Synthesis of Amides and Hydrazides (Acidic Component Varied)

Acidic component	Basic component	M.p. of anilide or phenyl-hydrazide*	Yield of anilide or phenyl-hydrazide
		°C.	*per cent*
Carbobenzoxyglycine	Aniline	144	80
Benzoylglycine.......	"	212	70
N-Phenylcarbamylglycine.... .	"	214	10
Benzenesulfonylglycine.........	"	151	5
Carbobenzoxyglycine	Phenylhydrazine	144	90
Benzoylglycine............. ...	"	185	95
p-Toluenesulfonylglycine........	"	169	80
Carbethoxyglycine....	"	123	65
Benzenesulfonylglycine.........	"	165	60

* Determined on recrystallized products giving satisfactory analyses.

butyric acid, 27.0 mg. of the amino acid, $[\alpha]_D^{23} = 16.0°$, were dissolved in varying amounts of N hydrochloric acid and the solutions made up to 2 ml. (Table II).

Action of D-Amino Acid Oxidase upon D-Threo- and D-Erythro-α-amino-β,γ-dihydroxy-n-butyric Acid[9]—The D-amino acid oxidase was prepared according to the directions given by Negelein and Bromel (8); 300 gm. of dried product were obtained from 1.6 kilos of lamb kidney.   70 gm. of the above preparation were stirred, for 45 minutes, at 38°, with 1.4 liters of 0.0167 M sodium pyrophosphate, pH 8.3.   Disodium phosphate was then added until the solution was 0.0167 M in respect to sodium pyrophosphate and 0.2 M in respect to disodium phosphate.   In each experiment, 2 ml. of enzyme solution, 0.3 ml. of water, and 0.37 mM of amino acid were used, and the oxygen uptake, at 30°, was determined in the usual manner (Table III).

*Some Observations on Enzymatic Synthesis of Amides and Hydrazides—*In one series of experiments 50 ml. of solution, adjusted to pH 4.7, containing 1 gm. of the acidic component, a 10 per cent molar excess of aniline

TABLE V

Enzymatic Synthesis of Amides and Hydrazides (Basic Component Varied)

Basic component	Acidic component	M.p of amide or hydrazide*	Yield of amide or hydrazide
		°C.	per cent
p-Toluidine....................	Carbobenzoxyglycine	162	90
Benzhydrazide†...............	"	162	80
m-Phenylenediamine†..........	"	155	40
α-Methylphenylhydrazine.......	Benzoylglycine	163	45
Hydrazine†....................	"	256	20

* Determined on recrystallized product giving satisfactory analyses.
† In these cases diacyl amides or hydrazides are formed.

or phenylhydrazine, 50 mg. of cysteine hydrochloride, and 25 ml. of papain-buffer solution,[7] were incubated for 10 days at 40° (Table IV).

In a second series of experiments 50 ml. of solution, adjusted to pH 4.7, containing a 10 per cent molar excess of the basic component, 1 gm. of carbobenzoxyglycine or hippuric acid, 50 mg. of cysteine hydrochloride, and 25 ml. of papain-buffer solution,[7] were incubated for 10 days at 40° (Table V).

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FATTY LIVERS AND GLUCOSE TOLERANCE IN THE WHITE RAT

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The production and prevention of fatty livers by dietary means have received extensive attention in recent years. On the basis of data published from the several laboratories working in this field, it is now possible to design diets which will produce levels of liver fat varying from 5 to 40 per cent of the moist tissue (1). With the aid of such diets, certain aspects of fat metabolism may now be investigated which formerly could be studied only with the use of poisons and anesthetics.

During the course of our studies on the relationship of diet to the production of fatty livers, it occurred to us that the carbohydrate metabolism of such animals might be profitably investigated. Studies of this type seemed of importance for two reasons: first, it has been suggested that the abnormal glucose tolerance of certain obese diabetics may be related to an increased fat and decreased glycogen content in the liver (2); secondly, it has been shown that a diabetic type of glucose tolerance curve is obtained after a short period of high fat feeding in man (3).

The present communication deals with observations made on the glucose tolerance and the glycogen content of the livers of rats receiving diets which were low in choline, and which contained varying quantities of fat, carbohydrate, and protein.

Procedure

In Series A, white male rats of the Wistar strain weighing approximately 120 gm. were used. In Series B, larger animals of approximately 150 gm. in weight were used to facilitate the collection of blood samples. For 1 week after arrival in the laboratory the animals received Purina rabbit chow; they were then placed in individual cages and received the experimental diets and distilled water *ad libitum*. The daily food intake and weight changes were recorded. The composition of the diets is given in Table I.

Glucose Tolerance—Glucose (3.5 gm. per kilo) was administered by intra-

peritoneal injection following a 16 hour fast. The blood samples were drawn directly from the tail into 0.1 cc. pipettes. The "true" blood sugar was determined by the method of Somogyi (4) on filtrates prepared by the zinc hydroxide precipitation procedure (5). The glucose tolerance of some of the animals was determined at the start of the experiments. These are designated in Table II as normal controls. The animals were maintained on the experimental diets for 24 days before the glucose tolerance was determined. It has been observed uniformly that after such periods of time abnormally large quantities of lipids are present in the livers of animals fed diets low in lipotropic factors and high in fat or carbohydrate (1). For determining the tolerance of the animals, a different group (composed of an equal number from each diet) of from six to eight animals was studied each day during the latter part of the weeks designated in Table II. We

TABLE I

Composition of Diets

All diets were supplemented daily with 1 dried yeast tablet (400 mg.) and 2 drops of cod liver oil.

	Diet 1	Diet 2	Diet 3	Diet 4
	per cent	*per cent*	*per cent*	*per cent*
Casein.........	5	30	5	30
Agar...........	2	2	2 .	2
Salt mixture*......................	5	5	5	5
Starch......................	48	23	73	48
Lard.........	40	40	15	15

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

routinely completed both the collection of blood samples and the analyses the same day.

Liver Glycogen and Lipids—The experiments were continued for at least 7 days after the last determination of glucose tolerance to insure the absence of any liver changes which might result from the procedure. The animals were then sacrificed by decapitation and the livers removed, wiped clean of adhering blood, cut into small pieces, and dropped into hot 30 per cent potassium hydroxide. The time required for this procedure was 30 to 40 seconds. The liver glycogen was precipitated according to the directions of Good, Kramer, and Somogyi (6) and, after hydrolysis, its reducing power was determined by the method of Shaffer and Somogyi (7). The total lipids (fatty acids plus non-saponifiable material of the liver) were determined in the following manner. An aliquot of the alkaline solution used for the determination of glycogen was refluxed for 1½ hours, acidified, and extracted three times with ethyl ether. The residue obtained by evaporation of the combined ethereal extracts was reextracted with

TABLE II

Glucose Tolerance of Experimental Animals

All animals were fasted 16 hours. 3.5 gm. of glucose per kilo were injected intraperitoneally. The "true blood sugar" is expressed in terms of glucose per 100 cc. of blood. The figures in parentheses show the ranges of values with the individual rats.

N., normal control rats.

Diet No.	No. of rats	Fasting	Time after glucose administration				
			½ hr.	1 hr.	2 hrs.	3 hrs.	5 hrs.
					Series A			
N.	7	*mg.* 93 (65–140)	*mg.* 204 (154–283)	*mg.* 165 (123–240)	*mg.* 140 (110–218)	*mg.* 111 (92–162)	*mg.* 97 (83–134)
					4th wk.			
1	6	129 (85–161)	287 (270–348)	252 (225–290)	237 (185–291)	205 (158–371)	170 (113–385)
2	6	104 (72–171)	240 (207–273)	219 (149–322)	223 (156–275)	200 (148–262)	141 (99–241)
					Series B			
N.	10	95 (68–125)	230 (204–265)	179 (141–232)	150 (105–240)	114 (82–155)	91 (57–113)
					4th wk.			
1	8	120 (80–229)	414 (307–526)	233 (174–306)	175 (138–289)	152 (89–260)	111 (87–160)
2	8	111 (73–142)	253 (212–358)	192 (154–243)	145 (130–186)	122 (82–143)	98 (70–144)
3	8	104 (73–124)	205 (165–254)	159 (98–209)	144 (99–174)	138 (99–167)	92 (56–118)
4	8	117 (102–137)	225 (158–329)	150 (94–237)	141 (84–207)	108 (69–141)	78 (59–117)
					8th wk.			
1	8	208 (162–299)	339 (268–484)	283 (204–452)	211 (131–390)	203 (146–397)	151 (108–207)
2	8	115 (77–149)	318 (221–411)	241 (169–414)	157 (121–183)	132 (102–160)	108 (84–125)
3	8	110 (68–142)	228 (165–339)	192 (99–285)	135 (106–188)	110 (89–144)	87 (52–101)
4	8	95 (68–116)	263 (192–357)	190 (155–271)	129 (104–171)	117 (78–146)	90 (68–115)

petroleum ether (b.p. 30–60°). This petroleum ether solution was dried over anhydrous sodium sulfate, transferred to a weighed flask, concentrated to a small volume, and finally dried to constant weight.

Results

In Table II the determinations of the glucose tolerance of the animals on the four diets are summarized. The first point of interest is the marked similarity of the two groups of normal controls. These values indicate that the rat, under standardized conditions, exhibits a characteristic and duplicable response to glucose injection, an observation which has been made previously by other investigators (8, 9).

The glucose tolerance of the animals receiving Diet 1 was found to be distinctly less than that of the control animals or those receiving Diet 2.

TABLE III

Glycogen and Lipids in Livers of Unfasted Rats

The experiment on Series A was 35 days in length; on Series B, 72 days. The liver data are calculated on the basis of the moist weight and of 100 gm. of body weight. The figures in parentheses show the ranges of values with the individual rats.

Series	Diet No.	No. of rats	Food intake per day			Change in weight	Liver per 100 gm. body weight		
			Carbo-hy-drate	Pro-tein	Fat		Weight	Glycogen	Total lipid
			gm.	gm.	gm.	per cent	gm.	gm.	gm.
A	1	9	3.2	0.3	2.7	−4.4	3.60 (3.0–4 2)	0.168 (0.10–0.29)	0.471 (0.27–0.75)
	2	6	1.8	2.3	3.1	+35 7	3.05 (2.5–3.8)	0.052 (0.04–0.06)	0.133 (0.09–0.17)
B	1	8	3.5	0.4	2.9	−3.0	4.37 (2.7–5.5)	0.223 (0.13–0.27)	0.812 (0.41–1.21)
	2	8	1.9	2.5	3.4	+82.2	2.96 (2.6–3.4)	0.095 (0.06–0.15)	0.156 (0.11–0 24)
	3	8	7.0	0 5	1.4	+14.1	4.24 (3.8–5.2)	0.269 (0.16–0.36)	0.385 (0.24–0.67)
	4	8	4.8	3.0	1.5	+67.1	3.00 (2.4–3.4)	0.132 (0.07–0 21)	0.107 (0.09–0.13)

The latter group on the other hand exhibited a glucose tolerance intermediate between that of the control animals and those receiving Diet 1. The findings with regard to Diets 1 and 2 were similar in Series A and B, but the differences were more striking in Series B. In contrast the animals on Diets 3 and 4 had glucose tolerances quite similar to those of the normal controls.

The quantities of lipids and glycogen in the livers of the experimental animals are given in Table III. The rats on Diet 1 exhibited the typical fatty liver which follows the feeding of a diet high in fat and low in lipotropic factors. The liver fat of 0.133 gm. per 100 gm. of body weight in the

animals on Diet 2 was in the normal range, as was to be expected when the diet contained 30 per cent casein (10). This relationship of diet and the liver lipids was likewise found in Series B; the rats on Diet 1 showed a 5-fold increase in liver fat over those receiving Diet 2.

Diet 3 was low in lipotropic factors and high in carbohydrate. Best and Ridout (11) have shown that the feeding of this type of diet will also produce fatty livers in the rat. However, the level of liver lipids observed in animals kept on such diets was not as high as that found in animals fed diets rich in fat. The value for liver fat of 0.385 gm. in the animals receiving Diet 3 was distinctly higher than the 0.107 gm. found with Diet 4. Thus, in agreement with previously cited work, the livers of the rats receiving Diets 2 and 4 contained normal quantities of lipids, while in the rats receiving Diets 1 and 3 the level of the liver lipids was markedly above normal.

Inspection of the glycogen data shows that the livers which contained excessive amounts of fat also contained increased quantities of glycogen as compared with those of rats on the diets containing 30 per cent protein. The data suggest that, in these experiments, the glycogen was deposited independently of the liver fat. Kaplan and Chaikoff (12) in citing their studies and those of others have pointed out that it is "unnecessary to invoke a metabolic antagonism between fat and glycogen to account for the amounts of these substances present in the liver."

One of the purposes of this work was to relate changes in glucose tolerance to liver fat and glycogen. Newburgh and Conn (2) have suggested that the decreased glucose tolerance observed in some obese diabetics is due to the deposition of fat in the liver, which prevents that organ from removing glucose from the circulating blood as readily as it normally does. Sayers and Orten (13) have shown that in a "diabetic" strain of rats there is a decreased liver glycogen when the animals are compared to similar ones having a normal tolerance. However, in the present studies the decreased glucose tolerance of the animals on Diet 1 does not appear to be related to a loss of ability by the liver to store glycogen, for these same animals had a higher liver glycogen than those fed Diet 2. Moreover, the animals on Diet 3 had a high liver fat and liver glycogen; yet their glucose tolerance closely approximated that of the normal controls. These results indicate that, under the conditions of these experiments, neither the level of liver fat nor the level of liver glycogen had a direct effect on the glucose tolerance.

When the food intake data in Table III are correlated with the glucose tolerance, it is found that the most marked decrease in tolerance occurred in the animals which ingested large quantities of fat and small quantities

of lipotropic factors (Diet 1).   The ingestion of large amounts of both fat and protein (Diet 2) resulted in a slight decrease in tolerance.   When the fat intake was decreased approximately 50 per cent, as in the animals on Diets 3 and 4, the glucose tolerance was similar to that found in the normal controls.   It should also be pointed out that any liver damage, which may have resulted from the low protein intake on Diets 1 and 3, did not affect the tolerance, since the animals on Diet 3 had a tolerance as high as those fed Diet 4.

The apparent lack of a direct relationship between the carbohydrate intake and the level of liver glycogen suggests the presence of other factors which have an important influence on the storage of glycogen in the liver. The evaluation of these factors must be postponed until additional studies have been made.

These findings do not eliminate the possibility that the level of liver fat may have some influence on glucose tolerance, for, while there were significant quantities of glycogen in the fatty livers, the excessive lipid material present may have affected the rate at which the glycogen was stored and removed.   MacLean, Ridout, and Best (14) found that, while the liver is able to store appreciable quantities of glycogen in the presence of large amounts of fat, there was less glycogen stored in very fatty livers than in the livers of choline-fed animals when a certain quantity of sugar was absorbed.   We are at present investigating the storage and removal of glycogen from the fatty liver.

SUMMARY

1. Glucose tolerance studies were made upon rats with fatty livers produced by dietary means.   No direct relationship was found between glucose tolerance and the fat or glycogen content of the liver.   The most marked decrease in tolerance was observed in animals receiving a diet which was high in fat and low in lipotropic factors (40 per cent fat and 5 per cent casein).

2. Significant quantities of glycogen were present in the livers which also contained increased quantities of lipids.   The glycogen appeared to be deposited independently of the liver lipids.

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ELIMINATION OF ERRORS IN THE COLORIMETRIC ASSAY OF NEUTRAL URINARY 17-KETOSTEROIDS BY MEANS OF A COLOR CORRECTION EQUATION*

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The colorimetric assay of 17-ketosteroids is based upon the fact that these substances react with the m-dinitrobenzene-alcoholic KOH reagents to give pigmented solutions (1). For crystalline 17-ketosteroids in pure solution the extinction coefficient of these solutions in the green (E_G) accurately reflects the 17-ketosteroid concentration according to Beer's law (2). When this colorimetric assay procedure is applied to crude urine extracts, the E_G determination is not always an accurate index of the 17-ketosteroid content, because such extracts may contain other chromogens which contribute to the E_G value and hence cause an error of overestimation (2). It has been shown elsewhere that this error can be reduced by separating a portion of these interfering chromogens from the urinary 17-ketosteroids with the aid of Girard's Reagent T (2).

The possibility of using a color correction equation for eliminating these errors in assay has been suggested by Fraser *et al.* (3). The equation used by them was worked out by Gibson and Evelyn (4) for another problem. It is based in principle on Vierordt's theory that the respective concentrations of two pigments in solution may be determined by measuring the extinction coefficients of such a solution at two wave-lengths, provided the extinction-wave-length curves for each pigment are known.

As applied to the present problem, the symbols in the equation $(K_i \times G - B)/(K_i - K_s) =$ corrected reading (C) have the following meaning: The extinction coefficients obtained for solutions of urine extract-m-dinitrobenzene reaction products with a green (E_G) and a blue (E_B) filter are indicated by G and B, respectively. K_i represents the ratio $E_G{:}E_B$ for a sample of the interfering chromogens; K_s represents the ratio $E_G{:}E_B$ for samples of crystalline 17-ketosteroids. The corrected reading, C, represents a calculation of that portion of the total extinction coefficient

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in the green (G) which is due to urinary 17-ketosteroid reaction products alone.

The value for K_s is easily determined with pure solutions of crystalline 17-ketosteroids. Ideally, the value for K_i should be determined with a pure solution of a single interfering chromogen. Because the interfering "chromogen" of urine extracts is composed of a mixture of substances whose identity has not been established, it becomes necessary to ascertain the K_i value by indirect methods. Thus, the valid application of the equation to the present problem depends upon the selection of a reasonably pure sample of the interfering chromogens of urine extracts. Samples of the interfering chromogens of different urine extracts should react for practical purposes like a single known chromogen to give pigments with essentially constant K_i values. Moreover, it is necessary to show that these interfering chromogens do not influence the 17-ketosteroid color reaction or vice versa. Except for stating that K_i was determined with extracts containing "no steroid," Fraser et al. (3) did not present data which justified the application of the equation to this problem. The purpose of the present paper is to report data showing that the equation is valid for this analysis and that it essentially obviates the need for chemical purification of neutral urine extracts prior to colorimetric assay.

Methods

Urine extracts were prepared and assayed according to procedures described previously (2).[1]

EXPERIMENTAL

Selection of Samples of Interfering Chromogens—When crude urine extracts are treated with Girard's Reagent T, a major portion of the interfering chromogens appears in the non-ketonic fraction. These interfering chromogens give rise to colored products giving an $E_G : E_B$ value of approximately 0.5. A smaller portion of interfering chromogens is carried over into the ketonic fraction along with the 17-ketosteroids (2). In unpub-

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[1] Particular care should be taken that any ethyl ether used in the procedure is free from impurities such as peroxides. These impurities give strong color reactions with the m-dinitrobenzene-alcoholic KOH reagent. A 0.2 cc. aliquot of a 2 cc. absolute alcoholic solution of the residue obtained by evaporation of 200 cc. of ethyl ether on a boiling water bath should not give more color in the m-dinitrobenzene-alcoholic KOH reaction than 0.005 mg. of pure 17-ketosteroids. The impurities encountered in the ethyl ether can usually be removed completely by any of the standard procedures for eradicating peroxides, such as washing the ether with a 10 per cent solution of ferrous sulfate in 1 N sulfuric acid, followed by water washings.

lished experiments it has been found that these residual chromogens of the ketonic fraction are also apparently non-ketonic substances that give rise to colored products with the same $E_G:E_B$ value.

The data of Fig. 1 provide evidence which suggests that most, if not all, of the total chromogens in certain urine extracts consist of these "non-
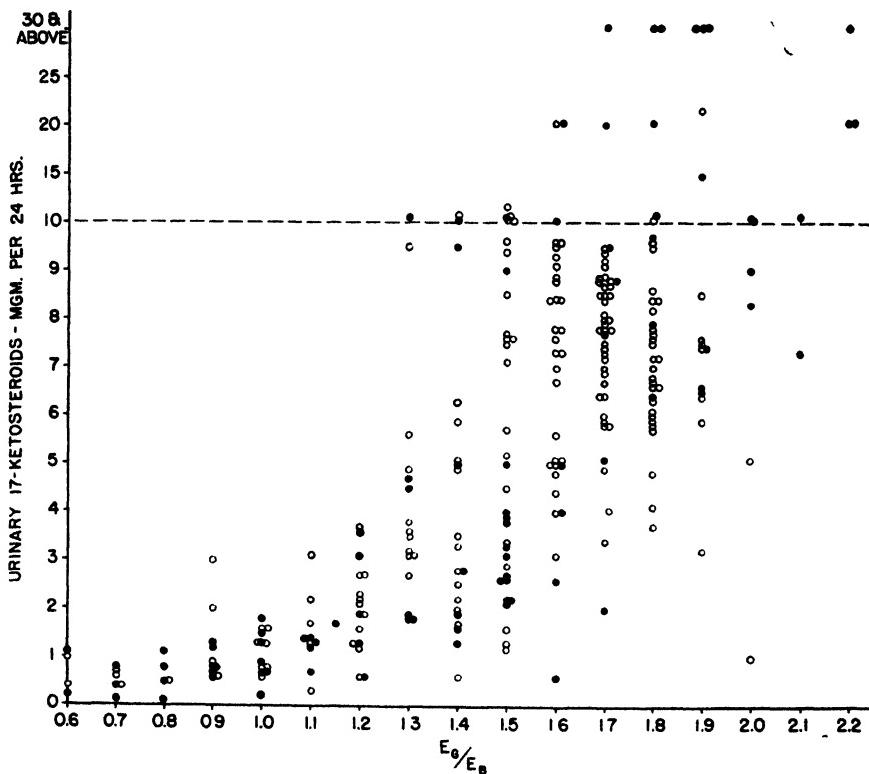

FIG. 1. The mutual relationship between the total uncorrected 17-ketosteroid values determined by colorimetric assay of partially purified ketonic fractions of 400 different 24 hour urine extracts and the color characteristics of the assay solutions as represented by the value $E_G:E_B$. The white circles are females, the black circles, males.

ketonic" substances. In Fig. 1 the values for $E_G:E_B$ of 400 urine extracts from normal and abnormal individuals of all ages are plotted against the uncorrected total 17-ketosteroid assay per day determined on partially purified ketonic fractions (2). It will be noted that the values for $E_G:E_B$ range between 2.2 and 0.6. There is a definite tendency for the lowest $E_G:E_B$ ratios to be associated with the lowest 17-ketosteroid assay values.

The lowest $E_G:E_B$ values observed (0.6) closely approximate the value (0.5) obtained with the non-ketonic chromogens. These low values were obtained on extracts of urine from young children and from panhypopituitary dwarfs. Such individuals presumably excrete little or no 17-ketosteroid. On the other hand, the highest values for $E_G:E_B$ (2.2) correspond to the value observed for pure 17-ketosteroids. They were obtained from patients who were suffering from corticoadrenal hyperplasia or individuals who had received injections of testosterone propionate. In these latter clinical situations the 17-ketosteroid output is known to be

TABLE I

Comparison of Determined and Theoretical Values for E_G and E_B before and after Addition of Dehydroisoandrosterone to Constant Amount of Pooled Interfering Chromogenic Substances

Experiment No.	DHA added	E_G		E_B	
		Determined	Theoretical	Determined	Theoretical
	mg.				
1A	None	0.048		0.078	
1B	0.017	0.108	0.109	0.105	0.106
1C	0.034	0.177	0.175	0.149	0.130
1D	0.073	0.276	0.287	0.181	0.180
1E	0.145	0.509	0.517	0.280	0.286
2A	None	0.128		0.194	
2B	0.024	0.211	0.219	0.240	0.231
2C	0.048	0.284	0.236	0.276	0.264
2D	0.096	0.426	0.378	0.347	0.329
2E	0.192	0.688	0.675	0.469	0.464
3A	None	0.076		0.111	
3B	0.046	0.233	0.227	0.191	0.180
3C	0.093	0.347	0.364	0.215	0.242
3D	0.139	0.502	0.514	0.284	0.309
3E	0.187	0.638	0.653	0.342	0.373
3F	0.280	0.886	0.930	0.438	0.499

high. This suggests that the actual 17-ketosteroid content of extracts with $E_G:E_B$ values as low as 0.6 is probably essentially zero. Consequently the $E_G:E_B$ value, 0.6, should approximately represent the color characteristics of a "pure" solution of the interfering chromogens of urine extracts.

Behavior of Interfering Chromogens in Color Reaction—In order to find out whether the interfering chromogens alter the characteristic color reaction between 17-ketosteroids and the *m*-dinitrobenzene-alcoholic KOH reagents and vice versa, the *G* and *B* values were obtained for samples of the interfering chromogens to which known amounts of crystalline dehydroiso-

androsterone-17 had been added. The determined values for G and B (Table I) may be compared with the theoretical values. The latter were calculated by adding to the G value and B value obtained with interfering chromogens alone the G and B values corresponding to the amount of pure dehydroisoandrosterone-17 added. The results show that the determined and theoretical figures agree reasonably well within the limits of experimental error. These experiments thus indicate that the reactions of
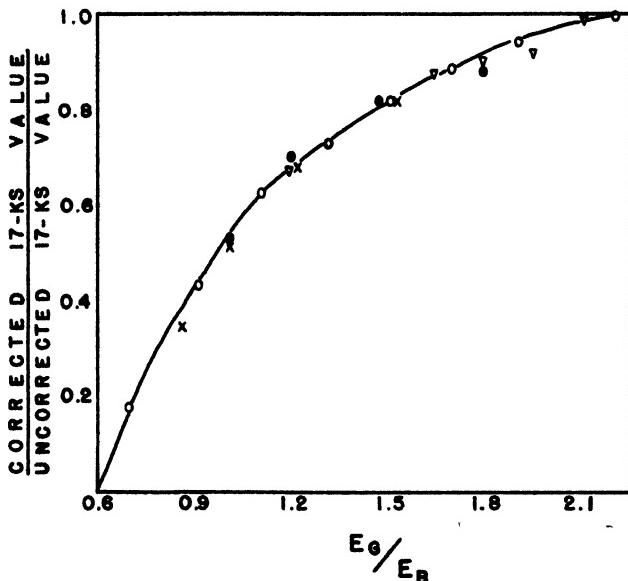

Fig. 2. The mutual relationship between the ratio for corrected to uncorrected 17-ketosteroid assay values and the value for $E_G:E_B$. The white circles describe the curve which is calculated from the color correction equation. The crosses, black circles, and triangles represent points determined by Experiments 1, 2, and 3, respectively, of Table I.

17-ketosteroids and of the interfering chromogens with the m-dinitrobenzene reagents are the same in mixtures as in separate solutions.

Accuracy of Color Correction Equation—With the aid of Gibson and Evelyn's equation, the value of the corrected 17-ketosteroid assay (C) has been calculated for theoretical solutions with a constant G value and $E_G:E_B$ values ranging between 0.6 (K_i) and 2.2 (K_s). The ratio of the corrected (C) over the uncorrected (G) values plotted against the respective $E_G:E_B$ ratio of each theoretical solution gives the curve described by the white circles in Fig. 2. The other symbols in the figure represent similar values determined experimentally (Table I). It was assumed that

the pooled samples of interfering chromogenic substances contained no 17-ketosteroid. Thus the ratios for corrected to uncorrected 17-ketosteroid values in experiments designated A is zero. In the experiments designated B to F, the ratios for corrected to uncorrected 17-ketosteroid contents were determined by dividing the value corresponding to the known amount of pure 17-ketosteroid added by the value determined by the uncorrected reading G. The E_G and E_B values for each experiment were used to determine the $E_G:E_B$ ratio. The closeness with which the theoretical
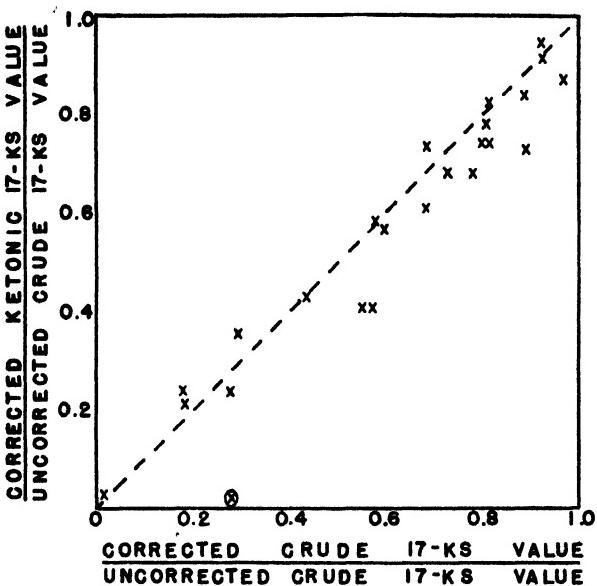

FIG. 3. Comparison of results obtained when the color correction equation is applied to assays made on crude and on partially purified extracts obtained from the crude extract, respectively. The common denominator is the uncorrected value for the crude extract. The interrupted curve represents the points where all values would fall if the two corrected values were identical.

and experimental values approach the same curve substantiates the validity of the equation and the theoretical considerations upon which it is based.

Substitution of Color Correction Equation for Partial Purification Procedure—The ratio of corrected to uncorrected 17-ketosteroid assay values has been determined as outlined above on twenty-four separate crude neutral extracts of urine. Subsequently, the crude extracts were treated with Girard's Reagent T (2) and the corrected 17-ketosteroid assay value of the partially purified ketonic fraction was obtained. The abscissa of Fig. 3

gives the ratio of corrected to uncorrected 17-ketosteroid values determined for the crude extract. The ordinate represents the ratio of the corrected ketonic value to the uncorrected crude value. It will be seen that with one exception the two ratios locate points which fall reasonably close to the theoretical dotted line describing the ideal situation in which both ratios are exactly the same. In the exceptional case, marked by a circle, the correction on the crude value was insufficient. It was observed that this particular crude extract was unusually pigmented. These pigments, which are also known to cause an overestimation in the assay (5), were largely eliminated when the crude extract was purified with Girard's reagent.

Comments

It is clear from the foregoing experimental observations that the non-ketonic chromogens must be taken into account in the colorimetric assay of urinary 17-ketosteroids. Relatively speaking, they may be responsible for much greater errors in the quantitative assay of urinary 17-ketosteroids than any of the other factors which have been studied in this (2, 5) or other laboratories (6–9). The data presented here show that the error introduced by the presence of these substances can, with rare exceptions, be eradicated by means of the color equation of Gibson and Evelyn as suggested by Fraser *et al.* (3).

Application of the equation is easy once the values for K_i and K_s have been determined. If reagents and colorimeter light filters similar to those used here are employed, it should be possible to utilize the values for K described herein. It is more advisable, however, for each laboratory to determine its own K values. For this purpose, a crystalline 17-ketosteroid and a representative pooled sample of urine extract containing no 17-ketosteroid (such as a non-ketonic fraction from a crude urine extract) are all that is required. Once these are established, a convenient table listing the ratios of corrected to uncorrected 17-ketosteroid values (see Fig. 1) for the entire range of $E_G:E_B$ values may be drawn up for routine use. This table serves as a list of correction factors corresponding to each $E_G:E_B$ value.

SUMMARY

Evidence is presented which shows that interfering chromogenic substances may cause significant and variable errors of overestimation in the colorimetric assay of neutral urinary 17-ketosteroids. Except in unusual instances, these errors may be largely eliminated by means of a simple color correction equation without preliminary chemical purification of the crude neutral extract.

We are indebted to Dr. A. M. Butler for suggestions in the preparation of this paper.

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A MICROMETHOD FOR THE ESTIMATION OF CEREBROSIDES IN NERVE TISSUE*

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In the course of an investigation on the regeneration of nerves a more accurate method of estimating micro quantities of cerebrosides than those already available was found desirable. All of these methods are modifications of Noll's (1) procedure for the estimation of the galactose set free from the cerebrosides by hydrolysis. In spite of improvements (2, 3) this method is still open to much criticism, particularly with respect to the lack of specificity of reduction methods.

The author has preferred a colorimetric estimation of galactose. The carbazole reaction of Dische (4) was found suitable for this purpose because it offers, besides a high degree of sensitivity, the advantage of being specific. A method for the quantitative and separate estimation of some hexoses by means of this reaction has already been worked out by Gurin and Hood (5) and the author has, with some modifications, followed their directions. Special arrangements, however, must be made to remove fatty acids and glycerol set free by the hydrolysis of the lipids because these substances interfere with the reaction.[1]

Method

Alcohol-ether extract containing 0.2 to 0.6 mg. of cerebrosides is measured in duplicate into flat bottomed tubes (Jena, 2 × ½ inch) and evaporated on a water bath at 60°. Heating after evaporation to dryness is avoided. 0.5 ml. of distilled water is added and the sample is stirred and finely dispersed with a glass rod. 0.5 ml. of 2 N hydrochloric acid is added and the tubes are placed in a boiling water bath and closed with rubber stoppers. Heating is continued for 2 hours, which brings about complete hydrolysis of the cerebrosides. After cooling, the fatty acids are removed by transferring the liquid with suction to test-tubes (Jena, 4 × ¾ inch) by means of immersion filter sticks (B-2).

[1] During the work the author's attention was called to a paper by Brückner (6) recommending the use of the orcinol reaction for the same purpose.

The sides of the tubes are washed once with 0.5 ml. of slightly acid water without disturbing the precipitate of fatty acids, and the wash water is sucked over into the test-tube. *The filtrate must be perfectly clear.* The test-tubes are then placed in a desiccator in a tilted position together with alcoholic solutions of the galactose standard (suitable amounts of this are 0.05, 0.10, and 0.15 mg. of galactose), and dried *in vacuo* over concentrated sulfuric acid and sodium hydroxide. The dried samples are extracted for 1 hour under proper cooling with 1 ml. of ice-cold ethyl acetate. The ethyl acetate is decanted and the last traces are removed in a desiccator. In this way the glycerol is removed without measurable loss of galactose. Now 5 ml. of the ice-cold sulfuric acid reagent and 0.15 ml. of carbazole solution are added, and the tubes are gently shaken until the reagents are thoroughly mixed and then placed in the vigorously boiling water bath for 10 minutes, along with a blank of sulfuric acid and carbazole. After cooling, the samples are ready for photometry in the Pulfrich step-photometer (Filters S-53 and S-43 and 1 cm. cuvettes). The color of the Dische reaction under the conditions given above follows the Lambert-Beer law. Only the value E_{530} is used for the calculation of the galactose content, E_{430} being used in order to get the quotient $E_{530}:E_{430}$ which serves as a control as to the presence of galactose as the only hexose.

Assuming a mean of 22 per cent of galactose in the cerebrosides, the cerebroside content will be (calculated) 4.55 × galactose.

Reagents—

1. 2 N hydrochloric acid.

2. Acid wash water. 1 drop of 0.1 N HCl per ml. of distilled water.

3. Ethyl acetate. Much stress must be laid on the purity of this reagent. 10 volumes of commercial ethyl acetate are thoroughly shaken with 1 volume of saturated solution of potassium carbonate. The aqueous layer is drawn off in a separatory funnel and the ethyl acetate is distilled, the first distillate containing water being discarded. The solvent is then dried over calcium chloride and afterwards over phosphorus pentoxide and finally distilled over sodium. Only the fraction boiling at 77.1° should be used. The liquid is to be kept in the cold.

4. Sulfuric acid. 7 volumes of sulfuric acid, *pro analysi*, and 3 volumes of distilled water; to be kept in the ice box.

5. Carbazole solution. 0.5 per cent carbazole in absolute ethyl alcohol. Commerical carbazole is purified according to the directions given by Gurin and Hood (5).

Results

The method has been applied to pure cerebrosides prepared according to Page (7) and to a hot alcohol-ether extract of dried ox brain, defatted by means of acetone at room temperature. The results of these analyses are given in Table I.

The method has also been applied with equal success to hot alcohol-ether extracts of dried but not defatted nerve tissue.

The mean value of the quotient $E_{530}:E_{430}$ for pure galactose is 2.35 and for the lipoidal material it was 2.14. This difference is readily accounted for by the greater amounts of organic material in the latter which cause a slightly yellow color with the sulfuric acid.

The pure cerebrosides are found to have a galactose content of 21.3 per cent, which is in fairly good agreement with the calculated value (21.5 to 22.2 per cent).

TABLE I

Analysis of Galactose in Pure Cerebrosides and in Lipid Mixtures

	Galactose			Cerebrosides	
	Found		Calculated		
	mg.	*per cent*	*mg.*	*mg.*	*per cent lipid mixture*
0.3 mg. cerebrosides	0.0620	20.7			
0.5 " " ..	0.109	21.8			
1.0 " brain lipids	0.0395			0.179	17.9
1.5 " " " . ..	0.0610			0.278	18.5
2.0 " " " .	0.0835			0.380	18.9
2.5 " " " .	0.0990			0.450	18.0
3.0 " " " ..	0.124			0.562	18.8
1.0 " " " + 0.1 mg. cerebrosides ..	0.0630		0.062		
1.0 mg. brain lipids + 0.2 mg. cerebrosides	0.0840		0.083		
1.0 mg. brain lipids + 0.3 mg. cerebrosides .	0.105		0.104		

SUMMARY

A colorimetric method is given for the quantitative estimation of cerebrosides by use of the carbazole reaction of Dische. After hydrolysis of the cerebrosides the fatty acids and the glycerol are removed. The fatty acids are separated through their insolubility in water and the glycerol is taken up in ethyl acetate.

The method can be applied to 0.2 to 0.6 mg. of cerebrosides.

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THE DISTRIBUTION OF SODIUM, POTASSIUM, CALCIUM, MAGNESIUM, INORGANIC PHOSPHORUS, AND CHLORIDE BETWEEN THE BLOOD SERUM AND CELLS OF NORMAL INDIVIDUALS*

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The present study on the simultaneous distribution of sodium, potassium, calcium, magnesium, inorganic phosphorus, and chloride between the blood serum and cells of normal individuals was found necessary as a basis of comparison for a similar investigation in a group of schizophrenic patients. Since the work of Schmidt (1) in 1850, there have been accruing in the literature data on the distribution of the anions and cations between normal human blood serum and cells. However, of all the comparative studies reported there was found but one in which the distribution of all the elements here referred to had been determined on the same blood specimen (Hald and Eisenman, 1937 (2)).

EXPERIMENTAL

Contrary to the opinion of Hald and Eisenman who state that for cell analysis "The only valid procedure appears to lie in analysis of whole blood and serum with separate measurement of the volume of the red blood cells" (2), the present investigators found, in agreement with Oberst (3), that packing the cells by centrifugation and analyzing definite volumes of the cells gave more consistent results than the indirect method.

Twelve normals—physicians, laboratory workers, and nurses at St. Elizabeths Hospital—were used as subjects. Cubital blood was obtained from these individuals before they had breakfast. The blood was collected anaerobically over Hg ((4, b) p. 54) and defibrinated according to the technique of Eisenman (5). The defibrinated blood was then centrifuged under oil at a speed of 1500 R.P.M. for 1 hour. The serum and cells were then separated and definite volumes of each were analyzed for Na, K, Ca, Mg, inorganic P, and Cl.

The cells were very carefully measured out in Ostwald-Van Slyke

pipettes. The P and Cl were determined on the unashed material—P by the method of Fiske and Subbarow (6), Cl by the Van Slyke and Sendroy modification of the Volhard and Harvey method ((4, b) p. 837). For determinations of the basic constituents, both cells and serum were separately

TABLE I

Concentration of Minerals in Serum and Cells of Normal Individuals, Milliequivalents per Liter

Case No.		Na	Ratio*	K	Ratio	Ca	Mg	Ratio	Inorganic P	Ratio	Cl	Ratio
1	Serum	132.3	33.9	4.0	1	4.8	2.3	1.2	2.3	2.3	107.4	1.9
	Cells	3.9	1	83.0	20.8	0	1.9	1	1.0	1	55.9	1
2	Serum	131.6	9.8	5.5	1	4.4	1.9	1	1.8	1.4	104.0	1.7
	Cells	13.5	1	80.8	14.7	0	3.2	1.7	1.3	1	60.5	1
3	Serum	135.5	11.7	4.8	1	4.0	1.9	1	2.1		103.3	1.9
	Cells	11.6	1	81.8	17.0	0	3.2	1.7			53.7	1
4	Serum	128.4	9.8	5.5	1	4.1	1.8	1	2.2	1.1	103.7	1.7
	Cells	13.1	1	88.5	16.1	0	3.9	2.2	1.9	1	59.5	1
5	Serum	141.8	10.9	6.0	1	4.2	1.7	1	1.7	2 2	104.3	1.8
	Cells	13.0	1	93.8	15.6	0	3.9	2.3	0.8	1	59.2	1
6	Serum	136.6	8.7	5.5	1	4.0	1.6	1	2.9	2.6	101.8	1.7
	Cells	15.8	1	92.1	16.7	0	4.5	2.8	1.1	1	60.7	1
7	Serum	140.4	13.2	6.1	1	4.4	1.6	1	2.0	1.4	102.8	1.8
	Cells	10.6	1	85.8	14.1	0	4.4	2.8	1.4	1 .	57.8	1
8	Serum	139.4	9.6	5.3	1	5.6	1.7	1	1.7		102.6	1.8
	Cells	14.5	1	80.8	15.2	0	3.2	1.9	Trace		56.6	1
9	Serum	139.0	9.5	5.9	1	4.8	1.7	1	2.0		102.5	1.7
	Cells	14.6	1	88.8	15.1	0	4.1	2.4	Trace		59.9	1
10	Serum	140.2	9.9	5.0	1	4.4	1.5	1	2.4	3.1	100.0	
	Cells	14.2	1	84.8	17.0	0	3.4	2.3	0.8	1		
11	Serum	138.7	12.9	5.5	1	4.7	1.5	1	1.9		100.0	1.6
	Cells	10.8	1	82.3	15.0	0	3.2	2.1	Trace		63.5	1
12	Serum	143.3	9.0	4.5	1	5.0	1.3	1	2.3	4.6	102.7	1.8
	Cells	15.9	1	84.8	18.8	0	3.1	2.4	0.5	1	56.5	1
Range												
	Serum	128.4–143.3		4.0–6.1		4.0–5.6	1.3–2.3		1.7–2.9		100.0–107.4	
	Cells	3.9–15.9		80.8–93.8		0	1.9–4.5		0.5–1.9		53.7–63.5	

* Ratio of serum to cell content.

ashed according to the method of Hald (7) and analyses carried out on aliquots of the ash solution. Na, Ca, and Mg were determined according to Hald's technique (7); K by the method of Shohl and Bennett (8). In each case 5 cc. of serum and cells were ashed. The ash solution was made

up to 10 cc. and the following aliquots used: for sodium 2 cc. of the ash solution both of serum and cells; for calcium 3 cc. of the ash solution of serum and cells; for potassium 2 cc. of the ash solution for serum determinations; whereas, for cell determinations 2 cc. of the ash solution were diluted to 10 cc. and 2 cc. of that used for the final analyses; for magnesium the volume of solution after Ca precipitation was made up to 5 cc. and the Mg was determined on 4 cc. of the supernatant fluid.

TABLE II

Comparison of Our Data on Simultaneous Distribution of Various Minerals between Blood Cells and Serum with Data Reported by Other Investigators

The results are expressed in milliequivalents per liter.

		Na	K	Ca	Mg	Inorganic P	Cl
Hald (indirect) (2, 7, 9)	Serum	129 1 to 143.3	3.0 to 7.6	4.1 to 5.8	1.2 to 2.4	1.9 to 2.8	100.0 to 105.4
	Cells	10.0 to 27.1	71.8 to 101.7	0 to 1.4	3.5 to 6.2		
Composite (3, 10–21)	Serum	129.4 to 154.0	2.9 to 7.6	4.0 to 5.5	0.8 to 2.5	1.9 to 4.9	99.0 to 108.0
	Cells	−17.0* to 27.1	80.0 to 120.0	−0.6* to 1.4	1.9 to 5.5	?	51.0 to 56.0
Snyder and Katzenelbogen	Serum	128.4 to 143.3	4.0 to 6.1	4.0 to 5.6	1.9 to 2.3	1.7 to 2.9	100.0 to 107.4
	Cells	3.9 to 15.9	80.8 to 93.8	0 to 0	3.1 to 4.5	Trace to 1.9	53.7 to 63.5

* Negative results are possible, since the determinations were not made on cells directly but calculated from whole blood, plasma, and hematocrit values.

Results

A comparison of serum and cell values for the twelve individuals studied (Table I) shows Na, Ca, inorganic P, and Cl to be more concentrated in the serum than in the cells, whereas for K and Mg the reverse is true.

DISCUSSION

In order to facilitate, for the reader, a comparison of the data here presented with those of others, there are tabulated the ranges obtained by Hald as well as a composite of the values found by others studying the cell and serum concentrations of one or more of the constituents here referred to (Table II).

There appears to be no discrepancy between the results here presented and those of previous investigators. Since Hald and Eisenman (2) review quite comprehensively the literature on Na, K, Ca, Mg, and Cl distribution, only that of inorganic phosphorus will here be discussed. Is in-

organic phosphorus equally distributed between serum and cells as the majority of investigators believe ((4, a) p. 1099) or, as postulated by Buell, is it entirely absent as such in cells and found present only because of enzymatic hydrolysis of the organic phosphorus compounds (22)?

The present study revealed in every case less inorganic phosphorus in the cells than in the serum—in three cases there being so little in the cells as to be recorded as a trace, whereas in one case only 0.5 milliequivalent was found. Since there does exist the possibility of enzymatic hydrolysis during centrifugation and in the short interval before analysis, more rigidly controlled experiments in which the blood is kept cold during centrifugation are needed before conclusions can be drawn as to the presence or absence of inorganic phosphorus in cells.

SUMMARY

Data on the simultaneous distribution of Na, K, Ca, Mg, inorganic P, and Cl between the blood serum and cells of twelve normal individuals are here reported and compared with the results of previous investigators.

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STUDIES ON THE METABOLISM OF BRAIN SUSPENSIONS

I. OXYGEN UPTAKE

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The respiratory metabolism of brain tissue has been studied by numerous authors using slices and hashes prepared in various ways (see *e.g.* Quastel (1)). However, no complete study has been made of the effects of varying conditions on the respiratory activity of brain tissue. Without standardized conditions, satisfactory comparisons cannot be made of the respiratory activity of brain in the presence of different substrates or of brains from animals subjected to varying conditions. In the present paper the effects of a number of factors on brain suspensions and slices are discussed. It is shown that suspensions prepared under optimal conditions compare in respiratory activity very favorably with slices and have many advantages for metabolic studies. As a result of this study, a number of interesting effects of ions, osmotic pressure, oxygen tension, and added catalysts have been brought to light. In forthcoming papers, information obtained from this study is applied to the study of carbohydrate combustion by brain tissue.

Methods

Whole rat brains were weighed and homogenized in the required amount of medium, in the apparatus of Potter and Elvehjem (2). Amounts of suspension, 1 to 3 cc., which contained a total of 300 mg. (usually) of fresh tissue were used in the flasks of Barcroft differential respirometers and the total volume of fluid in the manometer flasks was always made up to 3 cc. When two or more similar tissue samples were to be homogenized in different media, the brains were either divided midsagittally or they were mashed together in a small dental mortar and weighed samples of the homogeneous mash were taken for homogenization.

Brain cortex slices were kept in glucose-containing saline until sufficient had been prepared from two to four brains. The slices were drained on washed fine linen[1] before being weighed on a torsion balance. Approxi-

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[1] A method suggested by Dr. W. C. Stadie.

mately equal amounts of slices from each of the brains, totalling 170 to 250 mg., were taken for each manometer vessel.

Ca-free Ringer-phosphate solution was prepared according to Krebs (3), with the omission of the $CaCl_2$ solution. "Isotonic" NaCl solution was prepared by mixing 100 cc. of 0.9 per cent (0.154 M) NaCl solution, 10 cc. of water, and 7.9 cc. of a mixture of 4 parts of 0.25 M Na_2HPO_4 and 1 part of 0.25 M KH_2PO_4. This solution contains 0.13 M NaCl and 0.017 M phosphate, pH 7.4; it is similar to Krebs' medium but contains somewhat less K^+ and no Ca^{++} or Mg^{++}. "Isotonic" phosphate, sucrose, glucose, and urea solutions were prepared similarly, 100 cc. of 0.13 M phosphate (mixed di- and monosodium salts, pH 7.4), 0.284 M sucrose, 0.284 M glucose, and 0.284 M urea, respectively, being used in place of the 0.9 per cent NaCl solution. For "hypotonic" solution, 100 cc. of water were used in place of the salt solution. Glucose, final concentration 0.2 per cent, was always added unless otherwise stated.

New Barcroft apparatus of Pyrex glass were used and, at first, experiments were frequently spoiled by alkali creeping over the top of the center tube into the medium in the flask. To prevent this, the lower end of the filter paper roll was inserted into a piece of glass tubing shorter than, and fitting easily into, the center tube containing the alkali. The alkali-soaked paper was thus kept upright and prevented from touching the side of the center tube. With this method, alkalinization of the medium never occurred, while a number of other methods tried, such as the liberal use of grease, were unsuccessful. The extra glass affects the rate at which temperature equilibrium is reached and, for an equilibration period of 7 minutes in the bath, it was necessary to have glass tubes and wet paper rolls also in the compensating flasks.

In many early experiments the apparent O_2 uptake during the first 5 minutes was lower than in the next 5 minutes. A certain equilibrium concentration of CO_2 in the fluid and gas space has to be built up before readings truly represent O_2 uptake, and the time taken to reach this condition is often longer than the usual equilibration period. The trouble was largely removed by passing 5 per cent CO_2 through the medium before homogenization.

Terms—The term *fresh weight* indicates a weight of fresh, unmoistened tissue. *Moist weight* refers to the weight of slices after draining. *Initial dry weight* indicates the dry weight of slices used in manometer flasks as estimated from their moist weight and the moist weight to dry weight ratio found on a separate sample of slices dried immediately after weighing. *Final dry weight* means the weight found when slices were removed from the flasks at the end of an experiment, rinsed, and dried. The Q_{O_2} values of Warburg and many other workers represent c.mm. of O_2 taken up per hour per mg. of *final* dry weight.

Results

Effects of Physical Conditions in Preparation of Suspensions—The most active initial respiration by brain suspensions was obtained when the fresh tissue was homogenized directly in isotonic medium at about 37° in a somewhat worn homogenizer for the least time necessary to produce a smooth suspension.   Mashing, prior to homogenization, lowered the activity of the suspension about 20 per cent.   Peters *et al.* (4) reported that the respiration of mashed pigeon brain was considerably higher if the mashing was done on a plate warmed to 38° rather than on a cold plate. We found that it made little difference whether the mortar used for mashing brain was kept at 38° or cooled in ice; if anything the cold mortar gave more active material.   But appreciably, 10 per cent, more active suspensions were always obtained if the homogenization was carried out at 37° rather than in a cold medium.   Use of a tight fitting homogenizer gave a suspension up to 10 per cent less active than if a rather worn instrument was used. When homogenization was continued for 5 minutes instead of the usual 1 minute, the activity was lowered about 13 per cent.

In any experiment in which more than one homogenization had to be carried out, as when halves of the same brain or samples of the same mash had to be homogenized in different media, the time after each homogenization until the beginning of measurements had to be kept constant and minimal even if this involved allowing the intact brain or mash to stand a while.   The loss of activity of whole brain or mash on standing at room temperature is quite small, 8 per cent or less in 30 minutes, while isotonic NaCl suspensions lose 23 to 28 per cent at 25-37°; once prepared, suspensions usually keep best in the cold, about 13 per cent loss at 0°.

There was no appreciable difference in the respiration rate whether glucose was present in the medium at the time of homogenization or was only added in the manometer flasks.

Osmotic Pressure Effects—The fact that higher respiration rates with breis are obtained in Ringer's solution than in plain dilute phosphate buffer solutions has been mentioned by other authors (see *e.g.* Stare and Baumann (5), Greville (6)).   Fig. 1, lower curve, shows that increasing additions of NaCl to hypotonic brain suspensions caused increases of respiration rate up to 65 per cent at the optimal NaCl concentration, about 0.08 M; the rate was depressed again somewhat by further increase in salt concentration. Considerably more striking effects were observed when the original homogenization medium contained salt.   The respiratory rate with tissue homogenized in medium containing NaCl increased as the NaCl was increased up to an optimal concentration about equal to that of isotonic saline, when the rate was about 400 per cent of that obtained in hypotonic medium; further moderate increases in salt concentration had little further

effect but the rate was depressed again in strongly hypertonic solution
(see Fig. 3, *B*).   The marked difference between isotonic and hypotonic
suspensions was also found with pure gray matter from rat and rabbit
brain.

Similar results were obtained with sucrose (Fig. 2), glucose, or phosphate,
in place of NaCl, while isotonic urea solution behaved very much like
hypotonic solution.
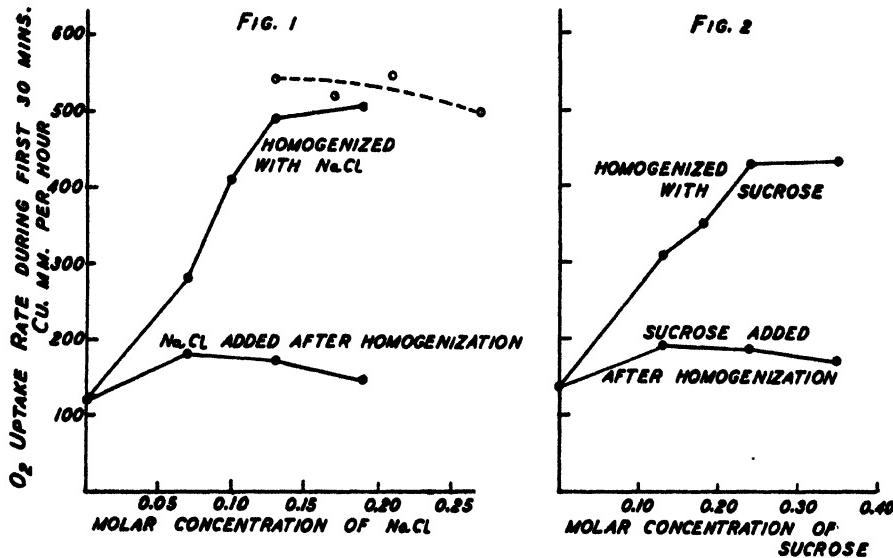
FIG. 1. Effect of NaCl on the initial respiration rates of brain suspensions (300
mg. of whole brain).   The brains of three rats were mashed together.   For the upper
solid curve, three 600 mg. samples were each homogenized with 3.4 cc. of 0.017 M
phosphate buffer, pH 7.4, containing varying amounts of NaCl; 2 cc. of each suspen-
sion and 1 cc. of the respective buffer-NaCl mixture were introduced into the manom-
eter flasks.   For the lower solid curve, one 1500 mg. sample was homogenized with
8.5 cc. of 0.017 M buffer without NaCl; 2 cc. samples were introduced into manometer
flasks which contained varying amounts of NaCl in 1 cc. of buffer.   The circles
(broken line) were obtained in a similar but separate experiment in which the higher
NaCl concentrations were used in the homogenization medium.

FIG. 2. Similar to Fig. 1 with sucrose instead of NaCl.   The scale of the abscissa
is adjusted so that equal distances on Figs. 1 and 2 denote equal osmotic pressures.

These results all emphasize the caution that no experiments to test the
effects of added substrates or catalysts should be carried out without adding
to controls enough non-specific material to make the effective osmotic
pressure of the control and experimental media equal.   An example of an
incorrect conclusion due to non-observance of this caution is pointed out
by Elliott and Greig (7).

We consider that the effects of homogenization in the different media are mainly due to the tendency of isotonic salts or sugars to prevent cell cytolysis, while in hypotonic medium cytolysis occurs. Cell disruption allows dispersion and dilution of respiratory catalysts with consequent decrease in respiration rate. Urea being freely permeable into cells would not be expected to prevent cytolysis.

Microscopic observations, kindly carried out for us by Dr. Balduin Lucké, were in fair accord with this theory. Homogenized tissue suspensions were examined unstained, stained with vital red, and stained with hematoxylin after fixation with formalin.

It was difficult to arrive at any conclusion by the examination of whole brain suspensions, since the small numbers of nerve cells were obscured by the large amount of myelin structures and other non-cellular material. Even in pure gray matter, the nerve cell bodies constitute only a small proportion of the total mass, but on comparison of suspensions of rabbit cortex, there appeared to be definitely less fragmentation of nerve cells in isotonic sucrose than in hypotonic medium. Occasional isolated branching ganglion cells and fair numbers of rounded up cells were visible in isotonic suspension, while in hypotonic suspension there were no branching cells, some apparently swollen rounded cells, and much more débris of all kinds.

In preliminary experiments it has been found that the initial respiration of liver suspensions shows effects of osmotic pressure similar to those found with brain. With liver suspensions, unequivocal histological observations could be made. Suspensions of liver in isotonic NaCl or sucrose showed very many cells, isolated and in groups, appearing well preserved, often polyhedral-shaped, and usually with distinct outlines. In hypotonic suspension far fewer cells were visible; they were swollen and poorly defined or fragmented. The condition of the cells seemed slightly better in sucrose than in NaCl medium. A suspension in isotonic urea medium appeared very like a hypotonic suspension, though the cells were slightly better preserved. A striking additional difference between suspensions in isotonic sucrose or NaCl, on the one hand, and urea or hypotonic solution, on the other, consisted in the coarse granularity of the cell cytoplasm in the former media and the poor definition of the granules in the latter media.

As may be seen in Fig. 3, the respiration of suspensions prepared in isotonic glucose or sucrose solutions was consistently better maintained than in isotonic NaCl. This suggests that the cells are better preserved in glucose or sucrose owing to lower permeability of the membranes to these substances than to NaCl. This apparent low permeability to glucose, coupled with the observation (to be published later) that the maximum rate of glucose oxidation by brain suspension is reached with glucose

concentrations as low as 6×10^{-4} M, suggests that the initial stage of glucose metabolism may take place at the brain cell surface.   The fact that tissue homogenized in hypotonic medium, in which the cells and their surfaces are presumably disrupted, does not oxidize glucose rapidly (Table VI), fits in with this idea.

As was the case with suspensions, slices in hypotonic medium respired initially less actively than in isotonic NaCl solution and the rate fell off more rapidly (Table I).   But, for reasons which are not clear, sucrose[2] was not able to maintain the respiration as well as NaCl.
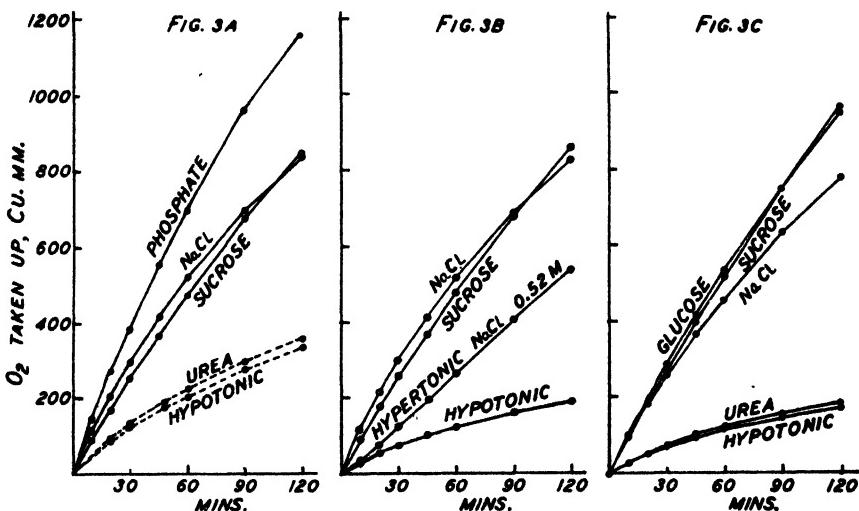
FIG. 3. The respiration of 300 mg. of whole brain homogenized in hypotonic, hypertonic, and various isotonic solutions.   For each set of curves (except the broken curves) two brains were mashed together and samples were homogenized, 300 mg. of tissue per 3 cc., in different media, all isotonic except where otherwise indicated. The two broken curves of Fig. 3, A were given by suspensions prepared by homogenization of left and right halves of the same brain without previous mashing.

The optimal concentrations of both NaCl and sucrose, when added to suspensions prepared in hypotonic medium, were lower than when present in the homogenization medium.   It seems as though osmotic pressure exerts two effects, one upon the cell integrity and another, with a lower optimum, apparently upon the enzymes themselves.   The effect of adding

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[2] Comparisons have to be made on the basis of the initial weight of the slices, since the final dry weight of slices after an experiment in a sucrose medium, even after being rinsed twice in water, is abnormally high.   The weight of sucrose in isotonic solution is 11 times that of NaCl in isotonic solution, and the solute is probably not readily washed out by brief washing.

NaCl was still pronounced even after complete disintegration of the tissue had been attempted by grinding with sand or freezing and thawing three times in hypotonic medium. The immediate effect of osmotic pressure on cell integrity in slices is probably less pronounced than with suspensions, so that the lower optimum due to the direct effect on enzymes was found also with sliced cortex. (But the rate in intermediate NaCl concentration fell off more rapidly than in isotonic NaCl.)

Specific Effects of Various Ions—Sodium chloride, phosphate, and other salts exert specific as well as osmotic effects. The initial respiration of suspensions prepared in isotonic NaCl was greater[3] than that of suspensions in isotonic sucrose; isotonic phosphate gave a more pronounced effect (see Fig. 3, *A* and *B*). The specific salt effects were clearly demonstrated by homogenizing the tissue in isotonic sucrose solution and adding varying amounts of isotonic salt solutions in the manometer flasks (Table II).

TABLE I

Effects of Different Media on Respiration of Brain Cortex Slices and Suspensions Prepared from Them

O_2 uptake rates, c.mm. per hour, by 300 mg. of moist tissue. All media contained 0.017 M phosphate. Oxygen in flasks.

		Hypo-tonic	NaCl, 0.07 M	NaCl, 0 13 M (isotonic)	Sucrose, 0.13 M	Sucrose, 0 24 M (isotonic)
Slices	First 30 min.	352	716	658	646	600
	2nd hr.	159	309	455	270	222
	3rd "	61	88	294	108	98
"	First 30 min.	339		655		
" homogenized	" 30 "	182		428		

The optimal final concentration of NaCl under these conditions was about 0.04 M. Sodium nitrate was equally active; equiosmotic amounts of sodium sulfate were more active, and of phosphate, still more active. These effects, which all disappear almost completely after an hour, cannot be solely due to the Na ions, since phosphate and sulfate gave effects greater than were obtained with the optimal NaCl concentration, but are presumably due to the anions. Since phosphate enters into carbohydrate metabolism, changes in its concentration might be expected to have special effects on brain respiration.

Ashford and Holmes (8) reported that chopped rabbit brain respired 45 per cent more rapidly in Ringer's solution containing 0.025 M bicarbonate in the presence of an O_2 and CO_2 mixture than in phosphate-Ringer's

[3] The initial acceleration due to NaCl is not found (Fig. 3, *C*) unless the experiment is set up quickly.

solution and O_2. With isotonic rat brain suspensions we found that the presence of bicarbonate in serum concentration had no greater effect than

TABLE II

Specific Effects of Salts

O_2 uptake rates, c.mm. per hour, during the first 30 minutes and during the 2nd hour (figures in parentheses) per 300 mg. of whole brain.

Homogenized in isotonic sucrose;* isotonic sucrose or salt solutions added to manometer flasks to give final concentrations of salts indicated

No salt	NaCl			NaNO₃, 0.043 M	Na₂SO₄		Extra phosphate		KCl, 0.043 M
	0.022 M	0.043 M	0.065 M		0.018 M	0.036 M	0.018 M	0.037 M	
580		728		718		818			626
(464)		(503)		(480)		(492)			(508)
496	542	596	570		552	616	576	648	
(439)	(435)	(459)	(441)		(441)	(451)	(435)	(456)	

Homogenized in isotonic NaCl;* isotonic solutions of salts added to manometer flasks to give final concentrations of salts indicated

0.13 M NaCl	0.043 M KCl, 0.087 M NaCl	0.043 M NH₄Cl, 0.087 M NaCl
772 (431)	724 (457)	618 (301)

Homogenized in isotonic solutions*				Homogenized in hypotonic solution*		
0.13 M NaCl	K-free 0.13 M NaCl	0.10 M KCl, 0.03 M NaCl	.	K-free	Salt solutions added	
					0.10 M NaCl	0.10 M KCl
520 (343)†		400 (281)	164†	166		
484 (291)†	504 (319)		†		190	160

No addition	Mg⁺⁺		Ca⁺⁺, 0.0025 M	
	0.0012 M	0.0025 M		
Isotonic suspension (homogenized in 0.13 M NaCl + 0.017 M Na-K phosphate)				
600 (343)	492 (352)	430 (320)	402 (335)	
630 (354)	520 (359)			
402 (150)	414 (161)	396 (173)	366 (187)	(No glucose)
Hypotonic suspension (homogenized in 0.017 M Na-K phosphate)				
226 (105)	222 (115)	236 (119)	142 (81)	

* All solutions contained 0.017 M Na-K phosphate buffer, as well as sucrose or salts, except in the experiments indicated, where K-free 0.017 M Na-phosphate buffer was used.

† Tissue mashed before homogenization for all marked series.

an equivalent small amount of phosphate. The reason for the disagreement with the results of Ashford and Holmes is not known.

Sodium bicarbonate, 0.155 M, and sodium phosphate, 0.10 M, solutions were added to 5.2 volumes of separate samples of isotonic NaCl-phosphate brain suspension. (The extra phosphate was added to the control to make the osmotic pressure and buffering capacity of the two suspensions about equal. Experiments showed that this amount of extra phosphate could cause only about 9 per cent increase in initial respiration.) The oxygen uptake of the suspension containing 0.025 M bicarbonate and 450 mg. of tissue in 3 cc. was measured in 95 per cent O_2-5 per cent CO_2 in a Dixon-Keilin apparatus; the respiration of the other suspension was measured in O_2 with CO_2 absorption in an ordinary Barcroft apparatus, readings being taken after an equilibration period at 38° equal to the time required to pass the gas through and equilibrate the Dixon-Keilin apparatus. The oxygen uptakes over 120 minutes, about 1100 c.mm., agreed almost exactly, in repeated experiments.

Serum contains 0.0025 M calcium and 0.001 M magnesium. The addition of 0.0025 M $CaCl_2$ to isotonic NaCl or hypotonic medium inhibited the initial respiration about 35 per cent but, especially in isotonic medium, the rate fell off with time much less rapidly than when no Ca was added, perhaps as a result of the tendency of Ca ions to decrease permeability and so to lessen osmotic disintegration of the cells. Addition of $MgCl_2$ had similar but somewhat less marked effects on isotonic suspensions. Mg causes citrate to stimulate the initial respiration of isotonic suspensions (see below). In the absence of glucose, and in hypotonic suspensions, Mg^{++} had no inhibitory effect (see Table II). Isotonic brain suspensions in "neutralized" (bicarbonate-free) rat serum (Warren (9)), containing 0.017 M phosphate, respired slightly less rapidly than suspensions in Ca-free Ringer-phosphate solution, the difference probably being due to Ca^{++} in the serum. The effects of Ca^{++} and Mg^{++} on suspensions are similar to those found by Dickens and Greville (10), and confirmed by us, on slices of cortex. Further, the average oxygen uptake in nine experiments on slices respiring for 90 minutes in isotonic NaCl-phosphate was 13.8 c.mm. per mg. of initial dry weight per hour, while the average of eleven experiments, reported by Elliott et al. (11), in medium containing Ca, Mg, and bicarbonate, was 9.8.

Potassium ions, compared with sodium ions, inhibit respiration somewhat (Table II). Omission of K^+ from the usual medium caused, if anything, a slight stimulation of respiration. Adding potassium chloride increased the activity of isotonic sucrose suspension very little. Replacement of part of the usual NaCl in isotonic NaCl solution by equivalent amounts of KCl lowered the respiration appreciably. KCl added to hypotonic suspension also caused less active respiration than did NaCl. Ashford and Dixon (12) found that addition of 0.1 M KCl to slices already in isotonic medium, thus making a hypertonic solution, caused large in-

creases in rates of respiration and aerobic glycolysis.   Dickens and Greville (10) confirmed this but found that KCl in isotonic medium lowered respiration; the accelerating effect was only to be found when extra KCl was added to already isotonic medium.   The effect of high KCl on slices and suspensions in isotonic media are therefore similar.   High KCl in hypertonic solution has not been tried on suspensions.   The respiration of isotonic suspensions containing some NH_4Cl fell off early.   Thus inhibitory effects occur with all cations tested, other than Na^+; whether Na ions exert specific effects is not clear.

The effect of hydrogen ion concentration has not been tested in this study.   At the end of 2 to 3 hour experiments, the pH, measured with a glass electrode, had usually fallen to about 6.6 in isotonic suspensions containing glucose, to about 6.9 in hypotonic suspensions, and sometimes to

TABLE III

Effects of Oxygen Tension on Respiration of Suspensions of Whole Brain and Slices of Cortex

		O_2 uptake, c.mm							
		Glucose present				Glucose absent			
		1st hr.	2nd hr.	3rd hr.	4th hr.	1st hr.	2nd hr.	3rd hr.	4th hr.
Isotonic* suspension, 300	Air	439	291	210	150	283	146	88	59
mg. whole brain	O_2	444	254	118	51	289	130	65	35
Hypotonic suspension, 300	Air	136	76	48					
mg. whole brain	O_2	151	70	35					
Slices of cortex in isotonic	Air	385	354	301	203	293	106	43	
NaCl per 300 mg. moist weight	O_2	548	411	242	81	284	83	36	

* In NaCl-phosphate.

6.1 with slices in NaCl-phosphate medium.   In the absence of glucose there was practically no change in pH.   With brain slices, Canzanelli *et al.* (13) found that the rate of oxygen uptake showed a marked optimum at pH 9 to 9.5 and only a small decrease when the pH was lowered from 7.3 to 6.0.

Effects of Oxygen Tension—Filling manometer flasks with pure oxygen instead of air did not change the rate of respiration during the 1st hour even with the rapid rates which occur with 450 mg. of tissue homogenized in glucose-containing NaCl-isotonic medium.[4]   During the 2nd hour, the rates in oxygen started to fall *below the rates in air* and during the 3rd

[4] This and the fact that increasing the rate of shaking did not affect the rate of oxygen uptake prove that the respiration rates observed in air in this work were not limited by inadequate diffusion of oxygen.

and 4th hours were only about 55 and 35 per cent respectively of the rates in air (Table III). Similar but less marked effects occurred in the absence of glucose and with hypotonic suspensions.

With slices of cortex, the initial oxygen uptake in air was evidently limited at first by inadequate diffusion of oxygen into the slices. But the respiration rate of the slices in air was better maintained and, after 2 hours, the rate in air was actually considerably greater than in oxygen. Similar results were obtained in the presence or absence of glucose. Ashford and Holmes (8) reported that chopped rabbit brain respired more rapidly in oxygen than in air. It is probable that, with the apparatus used by them and with the less finely divided tissue, inadequate diffusion of oxygen into the cells limited the respiration rates in air.

For tissue respiration studies in general, it may be better to use air rather than pure oxygen, provided the degree of subdivision of the tissue is fine enough, and other physical conditions (see Dixon and Elliott (14)) are such that diffusion of oxygen to the cells is adequate to maintain the full respiration rate in air.

These observations of the slowly developing inhibitory effect of high oxygen tension on brain tissue respiration may be related to the fact that prolonged oxygen breathing has adverse effects and that oxygen under increased pressure can produce convulsions in experimental animals and men (see *e.g.* Bean and Rottschafer (15), Bean and Bohr (16), and Behnke (17)).

Effect of Tissue Concentration—With isotonic suspensions, decreasing the concentration of tissue decreased the rate of respiration per unit weight of tissue only slightly or not at all. With hypotonic suspensions the decrease was appreciable, presumably because dilution of constituents of the respiratory system, which can occur more readily with cytolyzed tissue, has an appreciable effect (Table IV). The effects in the presence of heated liver extract are discussed in the following section.

Effects of Glucose and of Added Catalysts—With isotonic suspensions the respiration in the absence of glucose is initially somewhat lower than with glucose and falls off much more rapidly. Hypotonic suspensions seem largely to have lost the power to utilize glucose, the respiration not being greatly affected by its presence or absence in the medium. Suspensions homogenized in hypotonic medium and then made isotonic show intermediate behavior.

Examples of experiments on the effects of the addition of glucose and of substances which are concerned in the respiratory catalytic systems of Szent-Györgyi and Krebs and of coenzyme-containing tissue extract and of insulin are shown in Table V. References to work on the effects of these materials on other tissues, particularly pigeon muscle, may be found in a

previous publication (18).   Krebs *et al.* (19) have adduced evidence that
the citric acid cycle might occur in various tissues including brain.   From
the theories (see (18)) of respiratory catalysis by the C_4-dicarboxylic acids
and the citric acid cycle it might have been expected that citrate, fumarate,
and malate, or at least fumarate and malate, would give similar effects
with brain tissue.   Actually, however, the three substances behave dif-
ferently from each other and none behaves quite as fumarate does with
pigeon muscle.

Citrate, final concentration 0.007 M, added to brain homogenized in
isotonic medium containing glucose and Mg^{++}, caused an initial accelera-
tion of respiration of 12 to 23 per cent, which fell off after about an hour.
In the absence of Mg^{++}, the initial acceleration was much reduced or absent.
Whether Mg^{++} was present or not, the respiration rate was maintained

TABLE IV

Effects of Tissue Concentration on Respiration Rate

O_2 uptake rates, c.mm. per hour, per 100 mg. of fresh whole brain, during the first
30 minutes and during the 2nd hour (figures in parentheses).

Tissue in 3 cc..............	600 mg.*	400 mg.	200 mg.	100 mg.
Isotonic suspension†	160 (119)	155 (114)	153 (107)	147 (101)
Hypotonic " ‡	85 (38)	68 (42)	59 (38)	54 (34)
" " with 1 cc. liver extract‡...................	149 (36)	140 (45)	190 (45)	244 (58)

    * Measured in oxygen to avoid diffusion effects due to the high O_2 uptake rates
and somewhat viscous suspension.
    † Ca-free Ringer-phosphate.
    ‡ Graded small amounts of saline were added to the flasks to make the tonicity in
ιll equal.   1 gm. of tissue was considered equal to 1 cc. of isotonic saline.

better; *i.e.*, it was higher in the 3rd and 4th hour than in controls without
citrate.   In the absence of glucose, or with suspensions which had largely
lost activity toward glucose as a result of homogenization in hypotonic
medium, citrate had almost no effect or caused slight inhibition, whether
Mg was present or not.   It would seem that citrate or a derivative is to
some extent concerned in the metabolism of glucose by brain.   The initial
acceleration was apparently not due to removal of the inhibitory action of
Mg ions by the formation of an Mg-citrate complex, since (*a*) the respira-
tion rate in the presence of Mg plus citrate was somewhat greater than in
the absence of both and (*b*) Ca, 0.0012 M, inhibited respiration quite as
much in the presence of citrate as in its absence, though Ca and Mg
citrate complexes are believed to be formed to the same extent under
similar conditions (Hastings *et al.* (20)).

TABLE V

Effects of Citrate, Malate, Fumarate, Heated Liver Extract, and Insulin

O_2 uptake rates, c.mm. per hour, per 300 mg. of whole rat brain except where otherwise indicated.

Time	Glucose	No addition	Citrate, 0.007 M	Malate, 0.007 M	Fumarate, 0.007 M	Liver extract, 0.8 cc	Extract + malate	Extract + citrate	Insulin, 6 units	Insulin + extract + citrate
Homogenized in Ca-free Ringer-phosphate* (Mg present)										
First 30 min.	+	660	780	772		772	820	878	630	868
2nd hr.		461	500	460		425	448	527	450	512
4th "		259	361	232		192	200	306	256	282
First 30 min.	−	396	400	480		776				
" 30 "	+	320	Depancreatized cat brain (an-					388	320	368
4th hr.		232	terior portion of hemisphere)					273	232	263
Homogenized in isotonic NaCl-phosphate*										
First 30 min.	+	664	686	772	727					
2nd hr.		414	461	403	481					
3rd "		314	390	277	380					
First 30 min.	+	592	728	710	616	Mg, 0.0012 M, added				
2nd hr.		431	461	417	475					
3rd "		333	387	306	397					
Homogenized and tested in hypotonic phosphate†										
First 30 min.	+	336	344	472		520	586	512	328	526
2nd hr.		163	168	151		136	148	207	162	199
4th "		56	98	43		36	42	64	51	55
First 30 min.	+	240	256	390		488				
2nd hr.		117	104	115		98				
First 30 min.	−	206	202	330		488				
2nd hr.		84	72	81		97				
First 30 min.	+	192	170	282	252					
2nd hr.		116	97	103	129					
First 30 min.	+	208	202	298	264	Mg, 0.0012 M, added				
2nd hr.		122	119	109	139					
First 30 min.	+	144	Depancreatized cat brain (an-					200	146	182
4th hr.		64	terior portion of hemisphere)					80	54	81
Slices of rat cerebral cortex in isotonic NaCl-phosphate, per 300 mg. moist weight										
First 30 min.	+	612	670		656					
2nd hr.		439	428		430					
First 30 min.	+	518	640			Mg, 0.0012 M, added				
2nd hr.		425	405							
First 30 min.	−	366	382			Mg, 0.0012 M, added				
2nd hr.		126	107							
First 30 min.	+	492	492	562						
3rd hr.		231	239	241						

* All media in the flasks were adjusted by NaCl additions or omissions to be equiosmotic with and to have the same phosphate content as the Ringer-phosphate.

† Small additions of salt were made to bring all media in any one series to equal osmotic pressure. In the series which include liver extract, the final suspensions were nearly one-third isotonic.

$l(-)$-*Malate*, 0.007 M, caused a 15 to 20 per cent acceleration of the initial respiration of brain in isotonic medium.   The effect was only slightly less marked in the absence than in the presence of Mg ions and occurred in the presence or absence of glucose.   With hypotonic suspensions the initial acceleration was relatively more marked, 45 to 60 per cent, and intermediate effects occurred with suspensions to which salt was added after hypotonic homogenization.   With both isotonic and hypotonic suspensions, the acceleration due to malate rapidly disappeared and the respiration rate actually fell below that of controls without malate.   At constant osmotic pressure, the effect of malate addition was about doubled by increasing the concentration 10-fold; as with lower malate concentration, the acceleration fell off almost completely after an hour.   Though malate does not improve the maintenance of the rate of brain respiration, as fumarate does with pigeon muscle, it seems that at least part of its initial effect is catalytic for glucose oxidation, since, with suspensions prepared in hypotonic, glucose-free medium, the initial increase in respiration due to adding glucose plus malate was appreciably greater than the sum of the effects of the two added separately.   The later slight inhibition with malate may be due to an inhibitory effect of oxalacetate produced from malate.   Oxalacetic acid is known strongly to inhibit malic acid oxidation (21) and the lactic and succinic dehydrogenases (22).

Fumarate, 0.007 M, caused only a slight acceleration of respiration with isotonic suspensions but the rate was better maintained.   With hypotonic suspensions the initial acceleration was marked, though not so great as with malate, and the effect diminished with time, but it was still apparent after 2 hours; *i.e.*, inhibition did not set in as with malate.   The presence or absence of added Mg had no appreciable effect on the acceleration due to fumarate.   It seems possible that fumarate may be converted to malate, which then behaves like added malate, but that the malate-fumarate equilibrium is achieved only slowly in brain, so that the concentrations of malate and oxalacetate do not rapidly reach those obtained when malate itself is added.

With hypotonic liver suspension, Elliott and Elliott (23) found that the effect of added malate or related substances in increasing and maintaining the respiration rate was dependent upon the addition of sufficient univalent anion (normally chloride).   This specific effect was not found with brain. Malate and fumarate effects with brain were higher in salt-free medium (*i.e.* containing only 0.017 M phosphate) than when NaCl was present in the homogenization medium or added after homogenization.   Malate, and also citrate, accelerated the respiration in isotonic sucrose more than in isotonic NaCl.   In isotonic phosphate the effect of malate was decreased, and citrate became initially inhibitory.

The effects of citrate, malate, and fumarate on slices of cortex were very similar to their effects on whole brain suspension. Citrate caused considerable, 24 per cent, initial acceleration of respiration in the presence of Mg but little effect in its absence. There was only a transitory acceleration in the absence of added glucose, followed by some inhibition. There was no improvement in maintenance of rate in any case. Malate caused about 14 per cent increase in initial respiration (see also Greville (6)), the effect largely disappearing after 2 hours. Fumarate caused only a small increase in initial respiration rate.

Tissue extract was prepared by homogenizing fresh rat liver with an equal volume of water, heating to 90° for 15 minutes with stirring, and centrifuging. Determinations of the freezing point showed that such extracts had 0.8 times the osmotic pressure of isotonic saline. Extracts from skeletal muscle and brain gave effects similar to those of liver extracts but liver extracts were most conveniently prepared and seemed most active.

Liver extract, 0.8 cc., caused increases in initial respiration of about 20 per cent with isotonic suspensions, and up to more than 100 per cent with hypotonic suspensions. Smaller amounts of extract gave smaller effects. Since the extracts already contained glucose, the respiration rate reached the same level whether glucose was added separately or not. Adding malate or citrate with extract caused somewhat less than the sum of their separate effects. The respiration in the presence of liver extracts fell off rapidly, so that after an hour the rate was actually less than without extract. Either the extracts contain a progressive inhibitor as well as stimulators, or an inhibitor is produced by the action of the tissue on some constituent of the extract. The latter possibility is suggested by the curious fact that, in the presence of extract, the rate of oxygen uptake per unit weight of tissue decreased with increasing tissue concentration (see Table IV) as though larger amounts of tissue produced more inhibitor.

Added *insulin*[5] had no effect on the respiration of brain suspensions of rats, or of a cat[6] depancreatized 2 days previously, whether added alone or with various combinations of other substances in iso- or hypotonic medium. 6 and 24 units of insulin in 3 cc. were tried. MacLeod and Reiss (24) found that 0.1 per cent *metrazole* had no effect on the respiration of slices of rat brain cortex. With isotonic suspensions we found that 0.17 and 0.83 per cent metrazole caused inhibitions of 8 and 48 per cent respectively.

Comparison of Slices and Suspensions and General Considerations—As a method of preparing brain tissue for study, homogenization has definite

advantages over slicing. The preparation, draining, and weighing of sufficient slices for a series of experiments is delicate and tedious work. Duplicate oxygen uptake determinations on brain slices agree rather poorly (see duplicates (11)). This is probably partly due to variability in the activity of individual slices and partly to uncertainty in determining the amounts of tissue taken. The moist weight cannot be determined accurately, since slices cannot be thoroughly drained without being damaged, and final dry weights are variable owing to some disintegration during the experiment (see (11)).

Homogenization is simple, rapid, and allows accurate sampling, by pipetting, for respiration measurements and chemical analyses. With reasonable amounts of tissue, air may be used instead of oxygen, thus increasing convenience and avoiding the toxic effect of oxygen. Oxygen uptake measurements with large series of manometers can be started within 15 to 20 minutes after the death of the animals. Duplicate determinations on samples of the same suspension agreed within 2 per cent or less with uptakes of 300 c.mm. or over. When different halves of the same brain were homogenized separately in isotonic medium, with the same homogenizer, the widest variation was 7 per cent. However, there was considerable variation in the rates obtained with different animals (30 per cent between the highest and the lowest).

As was shown in previous sections, there seems to be no serious difference between isotonic suspensions and slices in the effects of various ions and other substances. Further, similar R.Q. values have been found with the two preparations. The respiration rate of whole brain homogenized in isotonic medium compares well with that of slices. Respiration rates, over the first 30 minutes, averaged 2210 c.mm. per hour per gm. of fresh weight for *whole* brain homogenized in isotonic NaCl-phosphate solution and 3100[7] for slices of *gray* matter in the same medium. White matter constitutes a large fraction of the whole brain and it is well known to respire considerably less actively than gray matter.[8]

However, the process of homogenization does cause appreciable damage to respiratory mechanisms. When *slices* of gray matter were homogenized in isotonic medium, the resulting suspension respired about 35 per cent less actively than did similar slices, unhomogenized, in the same medium

[7] Deduced from the rate per gm. of initial dry weight by dividing by 5. Possibly higher average rates for slices could be obtained if more nearly optimal conditions for their preparation were maintained. So far these conditions have not been worked out but preliminary observations suggest that the highest rates are obtained if only a few slices are prepared at a time and set up quickly, the slices being stored, until used, in glucose-containing Ca-free Ringer-phosphate solution cooled in ice.

[8] Pure cortex and rather impure white matter were separated from rabbit brain and homogenized in Ca-free Ringer-phosphate. The oxygen uptakes by 300 mg. of tissue in 90 minutes were respectively 545 and 265 c.mm.

(Table I). But by homogenizing the fresh tissue directly and starting measurements immediately, the loss of activity which must occur during
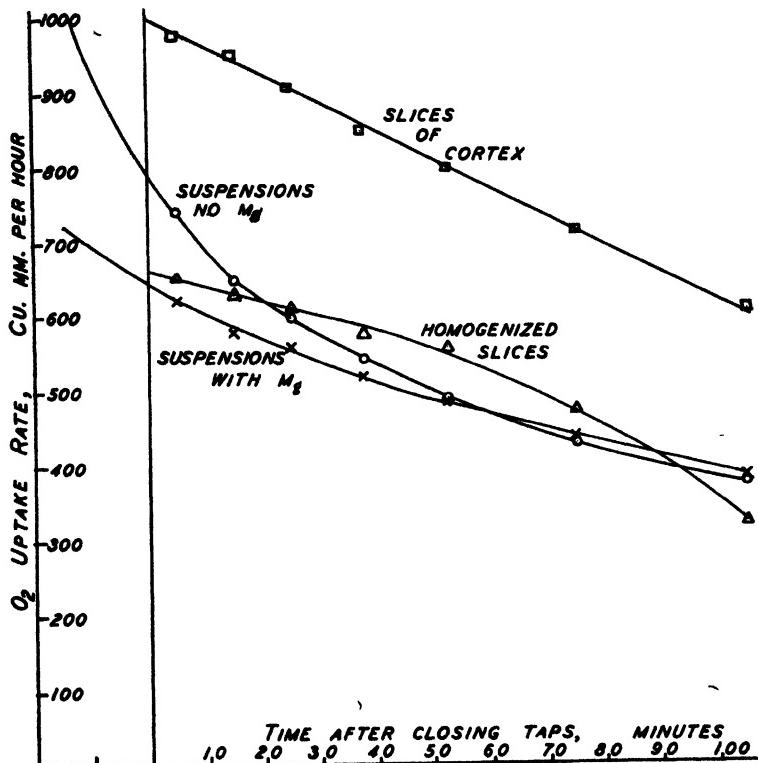

FIG. 4. Fall in respiration rate with time, with slices and suspensions. The rates are measured in c.mm. per hour for 300 mg. of fresh weight in glucose-containing medium. *Circles*, whole brain homogenized in isotonic NaCl-phosphate solution. Average of twenty-five experiments; time from starting homogenization till closing the manometer taps, 13 minutes. *Crosses*, whole brain homogenized in isotonic NaCl-phosphate solution containing 0.0012 M Mg (*i.e.* Ca-free Ringer-phosphate). Average of six experiments; time from homogenizing till closing the taps, 15 minutes. *Squares*, slices of cortex in isotonic NaCl-phosphate solution. Average of nine experiments; O_2 uptakes per 300 mg. of fresh weight are calculated by the following formula,

$$\text{C.mm. } O_2 \times \frac{300}{\text{moist weight used}} \times \frac{\text{moist weight to initial dry weight ratio (7.75)}}{\text{fresh weight to initial dry weight ratio (5.0)}}$$

Triangles, homogenized slices of cortex in isotonic NaCl-phosphate solution. Average of two experiments; O_2 uptakes per 300 mg. of fresh weight were calculated as above.

the time required for slicing and for draining and weighing the slices is avoided. The slicing itself probably does some damage.

As will be seen in Fig. 4, the rate of respiration of whole brain freshly homogenized in isotonic NaCl-phosphate medium fell off rapidly, about 10 per cent per 10 minutes, during the first 30 to 40 minutes, and thereafter less rapidly.   With slices of cortex and also with suspensions prepared from such slices, the extra rapid initial fall in rate did not occur.   This suggests that that part of the respiratory activity which falls off rapidly with fresh suspensions is lost during the long process of preparing slices.

Gerard *et al.* (25) studied the respiration of very small plugs of cat cerebral cortex in a special micro respirometer and observed a rapid decrease of respiration rate.   By extrapolating the curve of rate against time back to the time of taking the sample, they estimated the rate of respiration of cat cortex *in situ* to be 4000 to 5000 c.mm. per gm. per hour.   By extrapolating the time-rate curve (Fig. 4) for whole rat brain suspension in NaCl-phosphate medium back to the time of homogenization, the average original respiration rate was estimated to be about 3300 c.mm. per gm. per hour.   This figure, corrected for about 35 per cent loss of activity as a result of the mechanical action of homogenizing, might possibly represent the respiratory activity of the whole rat brain *in vivo*.   However, the highest rates of respiration found *in vitro* do not necessarily correspond most closely to the normal rates *in vivo*.   The extent to which the Ca and Mg of the blood are effective in influencing cell respiration *in vivo* is not known though only fractions of these ions are in diffusible ionic condition in the blood. It is possible that there may be special differences between brain in its normal physiologically active condition and the tissue which has been subjected to the abnormal injuries and stimuli of work *in vitro*.   For general studies *in vitro* on brain tissue metabolism, Ca-free Ringer-phosphate solution is possibly the most suitable medium and preferable to plain isotonic NaCl-phosphate solution containing no Mg ions.   Though the Mg ions lower the initial respiration slightly, the rate is better maintained, and the known importance of Mg^{++} in the catalysis of reactions involved in carbohydrate metabolism makes it seem dangerously unphysiological to omit this ion.

Homogenization in hypotonic medium definitely upsets normal respiratory activity, but it may be useful for observing the effects of the addition of certain substances to which the cell membrane is impermeable or of catalysts which may be destroyed or diluted below their optimal concentration by cytolysis.   However, the damage to structural features of the cell, which seem to be concerned in glucose metabolism, makes it improbable that the true functions of all added catalysts, etc., would be made apparent with cytolyzed tissue.   This damage does not necessarily inactivate catalysts concerned in metabolism but more probably simply

upsets their organization in space, thus changing their effective concentrations in limited localities and disrupting reaction series.

SUMMARY

1. Suspensions of brain tissue, prepared by homogenization in media made isotonic with salts, sucrose, or glucose, respire up to 400 per cent faster than suspensions prepared in hypotonic medium. Isotonic urea solution behaves like hypotonic medium. Additions of salt or sucrose to suspensions homogenized in hypotonic medium cause increases up to 65 per cent in the respiration rate.

2. The initial respiration of glucose-containing NaCl-isotonic brain suspensions is considerably decreased by small amounts of Ca^{++} or Mg^{++} but these ions improve the maintenance of activity. Mg^{++} has little effect in the absence of glucose or in hypotonic suspensions. The presence or absence of bicarbonate has no effect. Sodium chloride, sulfate, and phosphate have specific stimulating effects. Potassium ion is inhibitory.

3. Suspensions respire initially at the same rate in air as in oxygen. After an hour the respiration is progressively inhibited by oxygen. Oxygen inhibition can also be shown with slices of cortex.

4. Dilution of isotonic suspensions with isotonic medium has little effect on the activity per unit weight of tissue. The activity of hypotonic suspensions decreases somewhat on dilution.

5. Homogenization in hypotonic medium causes brain largely to lose the power of oxidizing glucose. Small additions of citrate to glucose-containing isotonic suspensions cause increases in the initial respiration rate which are dependent upon the presence of Mg. With or without Mg, citrate improves the maintenance of rate. In the absence of glucose and in hypotonic suspensions, citrate has no effect or inhibits slightly. Malate causes an initial acceleration of respiration, followed by some inhibition, in the presence or absence of glucose and Mg; its accelerating effect is larger with hypotonic suspensions. Fumarate slightly accelerates the initial respiration of isotonic suspensions and causes improved maintenance of rate. With hypotonic suspensions, fumarate causes considerable initial acceleration, but less than malate does and inhibition does not set in. Heated liver extract causes considerable initial increase in respiration, especially with hypotonic suspensions, followed by an inhibition. Insulin has no effect. Metrazole, in relatively high concentration, inhibits respiration.

6. The respiratory activity of slices of cortex and suspensions of whole brain is compared. It is shown that slices and suspensions behave in most respects in the same way and that isotonic suspensions are possibly

more suitable, and are much more useful, preparations for brain metabolism studies.

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ISOLATION AND PURIFICATION OF A SEROLOGICALLY ACTIVE PHOSPHOLIPID FROM BEEF HEART

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Numerous investigators have attempted to isolate the substance that confers on alcoholic extracts of beef heart the property of reacting with sera from cases of syphilis. Although a number of different methods have been employed, none has led to the preparation of any definite compound. Since the work of Noguchi (1), it has been recognized that the antigenic activity is associated with the acetone-insoluble portion of the extracts. One obvious method of approach, therefore, is the fractionation of the cadmium double salts of the phosphatides; yet there is considerable disagreement among published reports on cadmium chloride precipitation of beef heart extracts (2-5). Certain lecithin preparations, incompletely purified over the cadmium salt, have been described as antigenic *in vitro* (6, 7), although Wadsworth, Maltaner, and Maltaner (8) demonstrated that adequately purified lecithin and cephalin were without complement-fixing activity. The weight of evidence in the literature indicates that the active substance is precipitable by cadmium chloride, though separable from other similarly precipitable substances only with difficulty. It had been observed during earlier studies (9) in this laboratory that beef heart lipids from which all material precipitable by cadmium chloride had been removed were no longer active as antigens in the complement fixation test for syphilis.

This paper describes the isolation and purification of a new non-nitrogenous phospholipid from beef heart. For convenience in reference, it is proposed to designate this substance "cardiolipin." Serologic studies of cardiolipin were reported in a previous communication (10). It was found that a properly balanced mixture of cardiolipin with lecithin and cholesterol exhibits a specific complement-fixing activity with sera from syphilitic patients closely similar to the activity of beef heart extracts prepared for routine diagnostic use. No one of the three substances in this mixture has been found specifically active in the absence of the other two. Thus, it appears that the serologic activity of beef heart extracts is a function not of any single substance but of a system of at least three components, one of which is the newly discovered phosphatide.

Experimental Procedures

The use of fresh beef heart tissue was found to be essential; any procedure involving prolonged air drying of the tissue, as is customary in preparing antigenic extracts for routine diagnostic use, results in the formation of tarry products of decomposition that render the purification of the phosphatides extremely difficult. Moreover, the purified phosphatide, though stable in solution, is easily denatured in the dry state. Throughout the preparation, therefore, carbon dioxide was used to displace air, and the active substance was handled in solution or under layers of solvent. Yields were always determined by evaporating small aliquots of solutions, never by drying the entire fraction to constant weight; whenever practical, analyses were carried out on freshly dried aliquots of alcoholic solutions of known concentration. All materials were also protected from light whenever possible. For storage, the phosphatide is best kept in solution in absolute alcohol at 3–6°.

Extraction and Preliminary Fractionation—Fifteen fresh beef hearts were freed from blood, fat, and connective tissue as completely as possible, ground fine, and dehydrated by two extractions with acetone. For the first extraction, 1.2 ml. of acetone per gm. of minced tissue were used; for the second, 1.5 ml. per gm. In both cases the mixture was allowed to stand overnight at 3–6°, then filtered by suction, and the tissue washed once on the filter with acetone. The acetone extracts were discarded.

Without being dried, the tissue was next extracted twice with 1.5 ml. of 95 per cent alcohol per gm. of original weight. In each case the mixture was allowed to stand for 5 days at room temperature, with frequent shaking. A previous experiment had shown that only very little more of the active phosphatide could be obtained by a third extraction.

The first alcoholic extract was stored for 2 days at 3–6° and the precipitate of "white matter" was removed. To precipitate the phosphatides from the alcoholic solution an aqueous solution of cadmium chloride, containing 1 gm. per ml., was added gradually until further addition gave no increased precipitation. The mixture was allowed to stand overnight at 3–6° and the cadmium precipitate was collected and washed twice with 95 per cent alcohol. It was then suspended in 800 ml. of petroleum ether and the mixture was repeatedly extracted with 200 ml. portions of 80 per cent alcohol; petroleum ether was added whenever necessary to keep the volume constant. In this process lecithin passes into the alcoholic extracts, leaving the active substance in the petroleum ether layer (11). In order to test the alcoholic extracts for the presence of the cadmium salt of lecithin, small samples were aerated to remove petroleum ether and chilled if necessary to aid precipitation. After forty extractions, this test gave only a faint cloudiness, indicating that most of the lecithin had been re-

moved. The alcoholic extracts were reserved for the preparation of lecithin (11).

The petroleum ether solution at this stage contains rather large amounts of alcohol-soluble impurities which interfere with the complete removal of the cadmium salt of lecithin. The solution was therefore concentrated by distillation to about 75 ml. and precipitated with 800 ml. of alcohol. The precipitate was dissolved in 100 ml. of ether and the ethereal solution was poured into 10 volumes of alcohol, when the crude cadmium salt separated as a coarsely flocculent solid. This operation was twice repeated, and the precipitate was then redissolved in 300 ml. of petroleum ether. The solution was repeatedly extracted with 75 ml. portions of 80 per cent alcohol until the alcoholic extract gave no precipitate on being aerated and chilled in an ice bath.

The petroleum ether solution was concentrated by distillation and mixed with 10 volumes of alcohol in order to precipitate the cadmium salts. The precipitate was dissolved in chloroform. To remove cadmium, methyl alcoholic ammonia was added to the chloroform solution until the mixture was alkaline to litmus. There was no precipitate until the mixture was shaken with 10 per cent sodium chloride solution, when cadmium hydroxide separated and was removed. The chloroform solution was next freed from excess ammonia by washing with 10 per cent sodium chloride solution to which dilute hydrochloric acid was added drop by drop until the aqueous phase remained faintly acid to litmus after vigorous shaking. At this point a small amount of insoluble material separated at the interface between the solvent layers, and was discarded. The chloroform solution was washed with 10 per cent sodium chloride solution to remove excess hydrochloric acid.

The crude antigen concentrate thus obtained from the petroleum ether layer, after the removal of lecithin, is designated the P fraction.

The second alcoholic extract was worked up in the same manner, except that only four extractions with 80 per cent alcohol were required, since very little lecithin was present. The combined P fractions from the two extracts weighed 21 gm.

Preparation of Alcohol-Soluble P Fraction—The cadmium-free P fraction was obtained in alcoholic solution by the following procedure. The chloroform solution was concentrated by distillation, about 200 ml. of 10 per cent sodium chloride were added, and the remainder of the chloroform was removed by distillation under reduced pressure. The aqueous suspension was extracted with ether, and the ethereal solution was dried on sodium sulfate, filtered, concentrated, and poured into 10 volumes of absolute alcohol. The alcohol-insoluble portion was dissolved in ether, the ethereal solution was poured into absolute alcohol, and the precipitate that

separated was reprecipitated once more in the same manner. All the absolute alcohol solutions were then combined and concentrated to remove ether.

The alcohol-insoluble fraction was dissolved in methyl alcohol. Only traces of material insoluble in methyl alcohol were present.

Although the active phosphatide when purified is fairly soluble in absolute ethyl alcohol, it is persistently retained at this stage by the so called alcohol-insoluble fraction. Repeated precipitation from absolute alcohol is insufficient to effect a satisfactory separation. However, if the methyl alcoholic solutions are treated with sodium chloride, cephalin is precipitated, while the active phosphatide remains in the supernatant. This procedure is based on the method of Maltaner (12) for the purification of cephalin.

The methyl alcoholic solution of the "alcohol-insoluble" fraction was mixed with 2 per cent of its volume of saturated aqueous sodium chloride, and the precipitate which separated was triturated with absolute alcohol, dissolved in ether, and the ethereal solution poured into absolute alcohol. The methyl alcoholic supernatant from the precipitation with sodium chloride was concentrated nearly to dryness under reduced pressure, and the concentrated solution was diluted with a large excess of absolute alcohol. The insoluble residue was treated with ether, the mixture was centrifuged to remove salt, and the ethereal solution was poured into absolute alcohol. All absolute alcohol solutions were then combined, concentrated, and chilled in an ice bath to remove any remaining alcohol-insoluble material. All the alcohol-insoluble fractions were redissolved in methyl alcohol and again precipitated with sodium chloride, and the entire operation was repeated until no further appreciable amounts of alcohol-soluble material could be separated. By this method 8.9 gm. of the crude "alcohol-insoluble" P fraction eventually yielded 5 gm. of alcohol-soluble material, much of which proved to be the active phosphatide.

The total amount of the crude alcohol-soluble P fraction was 16.8 gm.

Purification of Cardiolipin—Cardiolipin was isolated from the alcohol-soluble P fraction by precipitating with cadmium chloride and purifying the resulting cadmium salt. To the alcoholic solution, an aqueous solution containing 0.5 gm. of cadmium chloride per ml. was added until precipitation was complete. The precipitate was collected after it had stood overnight at 3–6° and was washed once with alcohol and once with acetone. It was then dissolved in 100 ml. of ether and to this solution were gradually added 200 ml. of acetone. A white flocculent precipitate separated, leaving a yellow supernatant. After three more precipitations from ether by acetone, both precipitate and supernatant were completely colorless. With increasing purity, the cadmium salt had become less readily soluble in

ether; when ether was added, the salt first formed a transparent gel, which then dissolved very slowly.

For further purification the cadmium salt was dissolved in 50 ml. of benzene and precipitated by 150 ml. of ethyl acetate. After a second precipitation from 60 ml. of benzene by 120 ml. of ethyl acetate, the salt was washed twice with acetone and dissolved in 60 ml. of chloroform. When this solution was mixed with 30 ml. of alcohol, it became slightly cloudy and a trace of a chloroform-insoluble impurity was removed by centrifugation. The solution was then freed from cadmium, and the material recovered as before. In this case a colloidal precipitate formed immediately when ammonia was added to the chloroform solution of the cadmium salt, but it was necessary to shake the mixture with 10 per cent sodium chloride in order to cause flocculation of the cadmium hydroxide.

The purified cadmium-free phosphatide weighed 5 gm., a yield of about 0.4 gm. per kilo of fresh moist beef heart tissue. The steps in its preparation are presented in condensed form in the accompanying diagram.

Properties of Cardiolipin—The purified phosphatide was readily soluble in ether, petroleum ether, chloroform, ethyl acetate, and benzene, moderately soluble in methyl alcohol or in absolute ethyl alcohol, and slightly soluble in acetone. It was readily dispersed in water to a cloudy viscous solution, from which it precipitated as a white flocculent solid when sodium chloride was added to about half saturation. It could not be extracted from water by organic solvents in the absence of salt. An aqueous solution containing 15 mg. of the phosphatide per ml. had a pH of 6.0, determined colorimetrically with brom-cresol purple as indicator. In alcohol, the substance was neutral; 0.1425 gm. in 10 ml. of alcohol, with phenolphthalein as indicator, required 0.07 ml. of 0.1 N potassium hydroxide to neutralize; alcohol blank, 0.05 ml. of potassium hydroxide.

The phosphatide was optically active; $[\alpha]_D$ in alcohol = +7.0°. It gave a very weakly positive test for plasmalogen by the method of Feulgen, Imhäuser, and Behrens (13). It is worth noting that traces of plasmalogen are extremely difficult to remove; all other acetone-insoluble lipid fractions from beef heart, even the purified lecithin, gave strongly positive reactions for plasmalogen.

The iodine number of the purest cardiolipin so far obtained was 118. Earlier lots, made before the method of preparation had been fully developed, had iodine numbers varying from 101 to 108. These preparations may have suffered some oxidation; however, they were closely similar to the purest fraction in solubility and in serologic activity. The effects of more extensive oxidation, and the ease with which such oxidation takes place, are illustrated by the following experiment.

A portion of the alcoholic solution was evaporated to dryness on an

Preparation of Cardiolipin from Beef Heart Extract

Alcoholic extract + CdCl$_2$

- **Filtrate**
  Discarded

- **Ppt.**
  Dissolved in petroleum ether and extracted with 80% alcohol

  - **Alcohol extract**
    (Lecithin)

  - **Petroleum ether solution**
    Concentrated, pptd. from ether by alcohol

    - **Alcohol solution**
      (inactive impurities)

    - **Ppt.**
      Petroleum ether-80% alcohol separation repeated

      - **Alcohol extract**
        (Lecithin)

      - **Petroleum ether solution, P fraction**
        Freed from Cd and dissolved in absolute alcohol

        - **Alcohol-insoluble**
          Dissolved in MeOH and saturated NaCl added (1)

          - **Ppt.**
            Dissolved in ether, poured into absolute alcohol

            - **Alcohol-insoluble**
              Repeat from (1)

              - **Alcohol-insoluble**
                (Cephalin)

              - Alcohol-soluble

          - **Filtrate**
            Concentrated, dissolved in absolute alcohol

            - **Alcohol-insoluble**
              Repeat from (1)

              - Alcohol-insoluble
              - Alcohol-soluble

            - Alcohol-soluble

        - Alcohol-soluble

All alcohol-soluble fractions combined and reprecipitated with CdCl$_2$

- **Filtrate**

  Nitrogenous impurities not yet identified

  - **Ether-acetone mother liquors**

  - **Benzene-ethyl acetate mother liquors**

- **Ppt.**
  Dissolved in ether, pptd. with 2 volumes acetone

  - **Ppt.**
    Dissolved in benzene, pptd. with 2 volumes ethyl acetate

    - **Ppt. = Cd salt of pure cardiolipin**

electric plate in a stream of carbon dioxide, then dried overnight in a desiccator from which air had been displaced by carbon dioxide before evacuation. The dried material was no longer completely soluble in chloroform, ether, petroleum ether, or benzene. In distilled water it dissolved very slowly, giving a clear viscous solution which was acid to litmus. Most of the dried material dissolved slowly in 95 per cent alcohol, leaving a trace of fibrous residue insoluble in all the solvents tested. The iodine number of the alcohol-soluble portion had dropped to 74.5 from an original value of 103.2, and the serologic activity was greatly decreased. In general, preparations with iodine numbers lower than 100 have been found unsatisfactory for serologic use.

For combustion the phosphatide was dried *in vacuo* at 110°. Nitrogen was used to displace air before evacuation. Found, C 61.92, H 9.07.

Other analyses were made on freshly dried aliquots of an alcoholic solution. Since it was difficult to free such solutions from traces of sodium chloride, Cl was determined and all figures were corrected for the amount of sodium chloride present; percentages were calculated after the sodium chloride was deducted from the total solids in the solution, and the figure for sodium was corrected for the amount present as sodium chloride. Found, P 4.11, Na 2.72, Na:P = 1:1. Nitrogen, calcium, magnesium, and sulfur were absent.

Analysis of the cadmium salt indicates that one-half the sodium is displaced by cadmium. Found, C 57.74, H 8.20, Cd 6.33, Cl 1.83, P 3.98, Na 1.39, Na:P = 1.2, Na:Cd = 1:1.

Cadmium was determined by the method of Vornweg (14) adapted to semimicro quantities. Sodium was determined by the method of Darnell and Walker (15).

The chemical composition and properties of cardiolipin remained constant when it was subjected to repeated precipitation with cadmium chloride and repurification of the cadmium salt with benzene and ethyl acetate. The serologic properties were also unchanged by this treatment. The substance therefore appears to be reasonably pure.

Since cardiolipin has the composition of a sodium salt, the question arises whether the sodium was introduced by the use of sodium chloride. Preliminary experiments on this question suggest that at least a part of the active phosphatide in the crude extract is in the form of the sodium salt, since the final product contained sodium even when potassium chloride was substituted for sodium chloride throughout the preparation. The compound does not have the expected properties of a true salt, since it is recovered unchanged after solution in 0.1 N HCl, and attempts to prepare the free acid form of cardiolipin have so far been unsuccessful.

Hydrolysis—Cardiolipin was easily saponified by potassium hydroxide

in absolute alcohol. To a solution containing 0.48 gm. of cardiolipin in 40 ml. of absolute alcohol were added 8 ml. of 6 per cent alcoholic potassium hydroxide. A powdery precipitate began to form almost immediately. The mixture was allowed to stand at room temperature for 24 hours, when the reaction was apparently complete, as the supernatant remained clear. The precipitate was collected by centrifugation and washed eight times with absolute alcohol.

Fatty Acids—The combined alcoholic solution and washings were treated with carbon dioxide until no further precipitate formed, and the potassium carbonate was removed, thoroughly washed with absolute alcohol, and discarded. The alcoholic soap solution was concentrated under reduced pressure, diluted with water, acidified with hydrochloric acid, and extracted with petroleum ether. The petroleum ether solution was washed with water until the washings were chloride-free, then dried on sodium sulfate. The fatty acid fraction recovered on evaporation of the petroleum ether weighed 0.2992 gm., or 62.2 per cent of the original. It had an iodine number of 174.5 and an apparent molecular weight by titration of 326. Hydrolysis was evidently complete, since the fatty acid fraction contained no phosphorus.

Water-Soluble Fraction—The precipitate that had separated from the hydrolysis mixture was dissolved in 1 ml. of water and the solution made just acid to litmus with acetic acid, then mixed with 25 ml. of absolute alcohol. The gummy precipitate that separated was repeatedly triturated with absolute alcohol until it formed a powder, which was dried *in vacuo*. No alcohol-soluble substance other than a little potassium acetate could be found in the mother liquors. The alcohol-insoluble, water-soluble fraction weighed 0.1463 gm., or 30.4 per cent of the original. It contained 13.43 per cent of phosphorus, and gave a positive Molisch test. It did not reduce Fehling's solution until after it had been boiled for some time with 5 per cent sulfuric acid.

Acid Aqueous Fraction—The acid aqueous solution, after extraction of the fatty acids, was evaporated to dryness under reduced pressure. The salt residue was repeatedly evaporated with absolute alcohol to remove water and hydrochloric acid, then extracted twice with absolute alcohol. On evaporation the alcoholic extract left only 3 mg. of a brownish tarry residue. It may be concluded that no glycerophosphoric acid or other alcohol-soluble, water-soluble component is present.

DISCUSSION

No substance similar to cardiolipin has previously been obtained from animal tissue. However, phospholipids yielding carbohydrates on hydrolysis were discovered by Anderson and his coworkers in various acid-fast

bacilli (16) and certain of these carbohydrates were found to occur as phosphoric acid derivatives (17). A phosphorylated carbohydrate was also obtained on hydrolyzing the phosphatide from *Lactobacillus acidophilus* (18). The bacterial phospholipids appear to be more complex than cardiolipin; they contain traces of nitrogen and some of them at least are presumably mixtures rather than pure compounds. Nevertheless, the analogy between these substances and cardiolipin is extremely interesting, especially since the property of serologic activity is found among the bacterial phospholipids as well as in cardiolipin. Pinner (19) reported that the phosphatide prepared by Anderson from tubercle bacilli was serologically active.

Cardiolipin apparently represents a class of phospholipids not previously obtained in a pure state, compounds consisting of a polysaccharide phosphoric acid esterified with fatty acids. The number of different theoretically possible compounds of such a structure is obviously very great; not only may different carbohydrates as well as different fatty acids be present, but there is also a much wider possibility of isomerism, in the position of attachment of the fatty acids to the carbohydrate molecule, than is the case with lecithin and cephalin. This possibility of variation in structure may well be correlated with the capacity of some of these compounds for specific serologic activity.

SUMMARY

A new phospholipid from beef heart has been isolated and purified. On hydrolysis it yields fatty acids and a phosphorylated polysaccharide. The name cardiolipin is suggested for this substance, which is essential for the reactivity of beef heart antigens in the serologic test for syphilis. Chemical and serologic studies of cardiolipin are being continued.

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HISTOCHEMICAL CHANGES ASSOCIATED WITH AGING

I. METHODS AND CALCULATIONS*

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The recent development of methods of analysis and interpretation of data permits the histochemical description of some tissues in terms of their extracellular and intracellular components. From such a histochemical description of tissues of animals of different ages, it becomes possible to enlarge our knowledge of the aging process in animals. This is the subject of the present investigation.

The present paper is concerned with the analytical procedures and calculations used to determine the distribution of water, phosphorus, and potassium between the heart and skeletal muscle fibers and their environment. Paper II is a report of observations on the tissues of the rat from 30 days after birth to extreme old age, and the discussion of the changes in proportion and composition of the phases of the tissues as a function of age. In Paper III will be presented data on the effect of retardation of growth on the histochemical changes associated with aging.

In order to determine age changes in the phases of skeletal muscle, analyses have been made for fat, dry weight, collagen, elastin, chloride, total phosphorus, and potassium. In the case of cardiac muscle, concentrations of fat, blood, dry weight, collagen, chloride, total phosphorus, and, in some instances, elastin were determined.

These determinations were chosen in order that the intracellular concentrations of potassium, phosphorus, and water might be estimated. It was necessary to correct for the concentration of blood and neutral fat present in the tissue (1), and to calculate the proportion of extracellular fluid and of extracellular solids. The measurement of the extracellular fluid is based on the now widely accepted assumption that chloride exists exclusively in the extracellular fluid of muscle and some other tissues (1–3). Collagen and elastin presumably represent the extracellular solids. These primary data then permit the calculation of the concentration within the cells of such substances as total phosphorus, potassium, and water. This histochemical method of describing intracellular changes has found ap-

plication in the work of numerous authors; *e.g.*, Fenn (4), Yannet and Darrow (5), Muntwyler *et al.* (6), Eichelberger (7).

The specific intracellular constituents, potassium, phosphorus, and water, were chosen for study because (*a*) the *water content* of tissues during aging has long been of interest, since one of the oldest theories of the nature of senility ascribes to the organism a progressive desiccation; (*b*) the *phosphorus compounds* of tissue not only include many of the functional components of the fibers, but likewise represent a majority of the intracellular anions, whereas *potassium* is the major cation of the cells; (*c*) presumably, the greater part of the intracellular osmotic pressure is due to potassium ions and compounds of phosphorus.

Some of the methods used in our histochemical study are standard ones; others have been specifically devised or adapted for the present purpose of obtaining a histochemical description of a tissue on a small but uniform sample. These special procedures will be described in some detail.

Methods

Physiological Technique—The rats used were anesthetized with nembutal (Wistar group) or ether (Yale strain). The brief, light etherization employed is believed to have been without important consequence for the results. Blood was drawn from the heart after the chest was opened. Part of the blood was oxalated; the remainder was allowed to clot under oil, and the serum separated.

The heart was immediately removed and as rapidly as possible trimmed free of the auricles and valves. It was opened, blotted free of surface blood, and placed in a small stoppered bottle. Subsequently, when chilled, the tissue was minced. To prevent loss of moisture, this mincing was done in a cold room.

The skeletal muscle was taken from the abductor muscles of the thigh which were chosen because of their comparative freedom from fascia and tendon. Usually the hind legs were chilled for 10 to 15 minutes in ice water, after which the muscle was removed in the cold room, and carefully trimmed as free as possible of fat and fascia, and finally placed in a vial and finely minced.

Treatment of Tissue—The skeletal muscle was divided into three aliquots. One sample of 0.2 to 0.5 gm. was used for collagen and elastin estimation; another sample of about the same size was used for determining the dry weight, fat, and total phosphorus; and from a third, 1 to 3 gm., sample a nitric acid extract was prepared for the determination of chloride and potassium.

In some cases, the cardiac muscle was treated as described for skeletal muscle except that, since the total available tissue was less than a gm.,

smaller samples were used. Determination of the content of blood was made on the portion set aside for collagen and elastin measurement. In other instances, the minced tissue was divided into two parts instead of three, and dry weight, fat, chloride, and total phosphorus were determined on a single sample of myocardium.

Drying and Defatting—The dry weights were obtained by transferring 0.2 to 0.5 gm. samples of tissue to weighed 10 cc. Pyrex test-tubes, and heating for 15 to 20 hours in an oven at 100–105°. After being cooled in a desiccator, the tubes were reweighed. The dried samples were defatted by extraction with ethyl ether (1). The ether was added to the tubes and allowed to remain in contact with the tissue for 15 to 18 hours at room temperature. After the first portion of ether was decanted, a fresh amount of ether was added and left in contact with the tissue for an additional 3 or 4 hours. After this second portion of ether was decanted, the tubes containing the defatted tissue were returned for an hour to the oven at 100° and then reweighed.[1] The fat extracts were saved and returned to the samples which were later used for total phosphorus determination, or in some instances both chloride and total phosphorus measurements.

Estimation of Blood—Determinations of the blood content of the heart were made by mashing about 0.1 gm. of tissue with a rod after the addition of 1 cc. of 0.0125 M phosphate buffer at pH 7.2. After it had stood for 1 hour in the cold, the mixture was restirred, centrifuged 15 minutes at 3000 R.P.M., and the supernatant placed in the 1 cm. cell of a Koenig-Martens spectrophotometer. After brief aeration, the absorption at 576 mμ was observed and then corrected for the turbidity invariably present by measuring the absorption at 600 and 624 mμ. At these latter two wave-lengths, the hemoglobin absorption is less than 10 per cent of the maximum. To the absorption at 600 mμ was added the difference between the absorptions at 600 and 624 mμ; the resulting absorption represents the contribution made by the turbidity to the absorption at 576 mμ. This correction was then subtracted from the total absorption at 576 mμ, the remainder providing a measure of the tissue hemoglobin content. The absorption value so obtained was standardized in terms of hemoglobin concentration by making similar measurements on a sample of the animal's own blood, diluted 1:150. The assumption involved in this calculation is that the light absorption by the turbidity alone varies linearly between 576 and 624 mμ. Fig. 1 illustrates the principle used in making this correction and gives an example of the calculation used.

Unless corrections are made for the rather considerable amounts of myoglobin extracted with the blood from the *heart*, calculated concentrations of

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[1] This extraction suffices for the removal of neutral fat, but would be inadequate for the removal of all of the tissue lipids.

blood in the tissue will be too high. The suggestion of Watson (8) was followed in the use of the position of the α-band as a measure of the relative amounts of myoglobin. The position can be located rather precisely with the spectrophotometer as follows: The nicol prism is set to correspond to 1 or 2 per cent less than the maximum absorption. The *two* wave-lengths on either side of the maximum which correspond to this setting of the nicol are then determined. Half-way between these two wave-lengths is considered to be the position of the maximum. When the bracketing
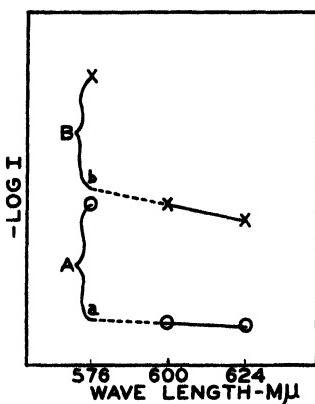
FIG. 1. Diagram of method for making turbidity correction in estimation of the blood in tissue. $\times$ indicates measurements of transmission at 576, 600, and 624 mμ with a turbid extract of heart tissue. O indicates similar values obtained with diluted blood from the same animal. The corrected "partial" absorption B may be compared with the "partial" absorption A. *Example:* Log $1/I$ for a 1:9.1 extract of sample of liver was 1.188 at 576 mμ, 0.437 at 600 mμ, and 0.360 at 624 mμ. The correction for turbidity at 576 mμ is therefore $2 \times 0.437 - 0.360 = 0.514$ which is equivalent to b in the figure. The net log $1/I$ for the solution is therefore $1.188 - 0.514 = 0.674$ ($= B$ in the figure) or $0.674 \times 9.1 = 6.14$ for the original tissue. For a 1:167 dilution of the rat's own blood, log $1/I$ was 0.749 at 576 mμ, 0.037 at 600 mμ, and 0.018 at 624 mμ. The "turbidity" correction at 576 mμ is therefore $2 \times 0.037 - 0.018 = 0.056$, the equivalent of a in the figure, leaving a net log $1/I$ of 0.693 for the dilute blood ($= A$ in the figure), or $0.693 \times 167 = 116$ for the original blood. The concentration of blood in the tissue is, therefore $(6.14 \times 1000)/116 = 35$ gm. per kilo.

wave-lengths are not more than 5 mμ apart and the slit width is not over 3 mμ, the mid-point is reproducible to ± 0.1 mμ. The value for the difference between the α-bands of rat hemoglobin and myoglobin has been taken as 3.7 mμ. This is based on several observations on the extracts obtained from previously perfused rat hearts.

In Table I is shown the recovery of blood added to liver and heart from which the original blood had been removed by perfusion. It will be apparent that, without correction for turbidity, the results would have been

much too high, whereas, after correction for turbidity (and in the case of
the heart for myoglobin as well), the blood present may be determined
with an accuracy of approximately 10 per cent.   When the tissue extracts
were prepared at room temperature, the recovery of added blood was only
85 or 90 per cent.

Repeated observations on skeletal muscle showed too little blood to
warrant routine spectrometric determination, but the blood content of the
heart is of such a magnitude that its estimation is of considerable
significance.

Collagen and Elastin—The methods of determining collagen and elastin
have been previously described (9).   The elastin values are quite low in the

TABLE I

Recovery of Blood Added to Perfused Rat Liver and Heart

The values are reported as gm. per kilo of tissue; the extractions were performed
at 6°.

	Blood found			Blood added
	Uncorrected	Corrected for turbidity	Corrected for turbidity and myoglobin	
Liver	56	4		0
	94	54		50
	114	69		68
	151	86		81
	235	128		119
Heart	75	59	51	43
	108	68	54	50
	108	79	68	63

tissues studied, being of the order of 2 gm. per kilo.   There is probably an
uncertainty of at least 1 gm. per kilo in both the collagen and elastin values.

Acid Extract—It was found that 10 volumes of 0.75 N nitric acid added
to 1 volume of tissue will give a protein-free tissue extract suitable for the
determination of both potassium and chloride.   Since such an extract
contains all of the acid-soluble phosphorus and presumably all of the in-
organic cations, it is comparable to an extract prepared with trichloroacetic
acid, and has the additional advantage that one may use it for the deter-
mination of chloride.   The substitution of nitric acid for trichloroacetic
acid permits chloride to be determined on the same tissue extract as that
used for the cations, thereby avoiding the variability usually encountered
through the use of separate aliquots of fresh tissue.   A comparison was
made of the analyses for potassium and chloride made on the nitric acid
extract and on the total tissue directly (Table II).   As an additional test

of the general applicability of this extract, values for acid-soluble phosphorus were compared, with nitric acid and trichloroacetic acid as the protein precipitants.   Rat liver and brain and dog skeletal muscle were analyzed for chloride by means of an open Carius digestion of the tissue followed by a Volhard titration.   The results were compared with those obtained from titration of nitric acid extracts of these same tissues following a brief digestion with nitric acid.   The potassium content of dog

TABLE II

Comparison of Analyses Made on Nitric Acid Extracts with Those Made on Trichloroacetic Acid Extracts (Acid-Soluble P), and with Direct Tissue Analyses (Cl, K)

The values are reported as mM per kilo of tissue.

	Cl		K		Acid-soluble P	
	HNO$_3$ extract	Total tissue	HNO$_3$ extract	Total tissue	HNO$_3$ extract	CHCl$_2$COOH extract
Skeletal muscle	20.2*	20.7*†	91.6*	90.3*†	65.7	66.2‡
	20.3*	20.3*†	91.7*	91.4*†	67.2	66.7‡
	20.4*	20.3*†		90.8	66.7	67.7‡
		20.3*†				
		20.3*†				
		20.3*†				
Average........	20.3	20.4	91.6	90.8	66.5 ·	66.9
Liver, rat	29.7*	28.8			40.7	39.8
	29.8*	29.8			38.3	37.2
	29.8*	29.0				
Average........	29.8	29.2			39.5	38.5
Brain, rat	39.6	38.9				
	38.4	38.7				
Average........	39.0	38.8				

* Aliquots of a single large sample.
† Dog.
‡ Rat.

skeletal muscle was measured both on whole tissue and on a nitric acid extract by means of the analytical procedure for potassium described below. The acid-soluble phosphorus was determined in rat muscle and liver by the method of Fiske and Subbarow (10); for each tissue both a nitric acid extract and a trichloroacetic acid extract were used.   From the data of Table II, it would appear that the nitric acid extract provides a satisfactory preparation for the determination of chloride, potassium, and acid-soluble phosphorus.

The adequacy of the protein precipitation by the nitric acid was checked by the addition of trichloroacetic acid to the extract obtained with nitric acid. There was only an additional 0.25 per cent of precipitate obtained, one-third of which was soluble in alcohol and appeared to be lipid.

Chloride—The chloride of skeletal muscle was determined by a Volhard titration of an aliquot of the nitric acid extract. To the sample of extract were added 1 cc. of 0.03 N silver nitrate per gm. of tissue represented and sufficient concentrated nitric acid to increase the volume of solution by 50 per cent. The sample was heated 10 or 15 minutes in a boiling water bath with the addition of a drop of 30 per cent hydrogen peroxide. Even without the addition of peroxide, the samples were nearly colorless.

After the digestion, an amount of 40 per cent ferric alum was added equal to one-eighth of the volume of the solution. The sample was then titrated with 0.01 N NH$_4$SCN, with a 3 cc. Rehberg type burette. The burette was provided with a 3-way stop-cock and reservoir for ease of filling. Titrations were performed in ice water to sharpen the end-point.

The chloride of *myocardium* was either determined as described above or, if the amount of tissue were not sufficient, it was determined on the sample of tissue used for total solids and fat determination. In this case, the procedure followed was essentially that described by Hastings and Eichelberger (1) except that the starting material was dried rather than fresh tissue. Serum chloride was determined on samples of 0.1 to 0.2 cc. by an open Carius digestion followed by the Volhard titration. The serum samples were measured from the Lang-Levy type of micro pipette (11) which will deliver 0.1 cc. with an accuracy of 0.1 per cent. The details of the serum analysis were similar to those given in full by Keys (12).

Total Phosphorus—The total phosphorus in *skeletal muscle* was determined by the Fiske and Subbarow method (10) on the solid left from the dry weight determination. For each gm. of tissue, 1 cc. of 10 N sulfuric acid was added and the sample digested with fuming nitric acid. The digestion was readily accomplished by heating a large number of tubes in a bath consisting of 75 per cent ammonium sulfate, 15 per cent concentrated sulfuric acid, and 10 per cent water. The bath temperature was increased from 140° at the beginning of the digestion to 180° at the end. Meanwhile, fuming nitric acid was added cautiously down the side of the tube. Fuming nitric acid oxidizes the sample more quickly than does ordinary concentrated nitric acid and with far less danger of spattering. Samples which proved difficult to digest were allowed to cool, a drop of 30 per cent hydrogen peroxide was added, and the tubes were then returned to the bath. Upon completion of the digestion, all of the samples were allowed to remain 1 hour in an oven at 110° to drive off the last traces of nitric acid or peroxide. The color was then developed with the Fiske and

Subbarow reagents and compared with suitable phosphate standards in the colorimeter.

The total phosphorus measurements in the case of the cardiac muscle were sometimes, of necessity, carried out on samples which had been previously used for dry weight and chloride estimations, and hence contained both silver and ferric alum. This caused the formation of a precipitate of silver chloride on the addition of the molybdic acid-bisulfite reagent, which contained a small amount of chloride as an impurity. The samples were easily filtered or centrifuged to yield clear solutions for comparison in the colorimeter. The standards used were prepared with an amount of ferric alum roughly equivalent to that added to the tissue samples during the chloride titration. As a check on the validity of this procedure, the total phosphorus of a muscle sample was estimated with and without the addition of ferric alum and silver nitrate. In the absence of these additional reagents, values of 67.8, 67.0, and 67.3 mM of total phosphorus per kilo of tissue were observed, whereas in the presence of the chloride reagents, the figures obtained for total phosphorus were 66.3, 66.3, and 66.3 mM per kilo, an average difference of less than 2 per cent.

Potassium—A volume of nitric acid extract representing 0.1 to 1 gm. of tissue was placed in a platinum crucible with 0.03 cc. of 10 N sulfuric acid. After evaporation in an oven at 60°, the sample was heated to 500–550° overnight in a muffle furnace. The evaporation was carried out at a low temperature, since, at higher temperatures, there was a tendency for minute droplets to spatter out of the crucible. The metaphosphate of the resultant ash was hydrolyzed by moistening the walls of the crucible with 0.1 to 0.2 cc. of concentrated HCl, followed by evaporation to dryness on the steam bath.

The following description applies to the subsequent treatment of a sample representing 0.5 gm. of tissue. For other quantities of tissue, proportionate volumes of reagents were used. The dried salts in the crucible were dissolved by adding 1.100 cc. of 0.1 N HCl. After thorough mixing, 1 cc., representing 90.9 per cent of the original sample, was withdrawn and transferred into a 5 cc. conical centrifuge tube. This was dried overnight in an oven at 100°. The salts in the tube were dissolved in 0.15 cc. of 3 N HCl and the potassium precipitated as the chloroplatinate by the addition of 2 cc. of 1 per cent $H_2PtCl_6 \cdot 6H_2O$ in alcohol. This reagent was freshly prepared from an aqueous solution of chloroplatinic acid containing 1 gm. of $H_2PtCl_6 \cdot 6H_2O$ per cc. by diluting it 100-fold with *absolute* alcohol. After thorough stirring, the tubes were allowed to stand ½ hour and centrifuged. The supernatant fluid was siphoned off as completely as possible. The precipitate was stirred up with 2 cc. of a wash solution consisting of absolute alcohol containing 2 cc. of the 1 per cent reagent per 100 cc.; *i.e.*,

containing 0.02 per cent $H_2PtCl_6 \cdot 6H_2O$. The washing solution was then removed by centrifuging and siphoning. In the event that the precipitate was adherent to the walls and showed a tendency to be drawn off with the original reagent or with the washing solution, the siphoning was interrupted, the walls were scrubbed with a blunt rod, and the tube recentrifuged before the supernatant liquid was removed. The tubes containing the precipitate were allowed to dry at room temperature for several hours, then heated to 100° for half an hour, and finally cooled and the precipitate dissolved in the tube in exactly 2 cc. of water.

An aliquot containing 4 to 5×10^{-6} equivalent, *i.e.* 0.2 cc. in the present instance, was transferred to a 50 cc. flask and exactly 25 cc. of the reagent described by Shohl and Bennett (13) were added. This consists of a freshly prepared solution of 0.4 N potassium iodide in 0.04 N hydrochloric acid conveniently made by mixing 4 volumes of 0.05 N hydrochloric acid with 1 volume of 2 N potassium iodide. The potassium iodide solution will keep for some time if preserved by being made up in 0.01 N sodium hydroxide. The acid-iodide reagent was allowed to stand 45 minutes with the chloroplatinate in order for the brown iodoplatinate color to develop. Comparison was then made in a colorimeter against a standard prepared at the same time and which contained 1 cc. of a 0.1 per cent solution of K_2PtCl_6 plus 25 cc. of reagent. The reading of the colorimeter was considerably improved by the use of a Wratten No. 61 filter (transmission maximum 535 mμ) placed in the eyepiece. With a 300 watt light source and the standard set at 40 mm., the readings were reproducible to within 0.1 mm.

By the above procedure, the potassium concentration of various standard solutions could be determined within 1 per cent of the theoretical value. The known potassium solutions tested consisted of either potassium chloride, potassium sulfate, or a salt mixture composed of potassium phosphate, sodium chloride, magnesium lactate, and calcium chloride in approximately the proportions encountered in muscle. These solutions were either analyzed directly or were first ashed in the muffle furnace after the addition of sulfuric acid. In either case, the potassium found agreed within 1 per cent with the amount known to be present.

Specific Gravity—The specific gravity of serum was determined according to the gradient tube principle first described by Linderstrøm-Lang *et al.* (14, 15).

The serum densities were determined in a gradient tube arranged as follows (Fig. 2): A 250 cc. graduated cylinder (*A*) was mounted in a larger cylinder or battery jar of the same height (*B*) and fixed to the bottom of the larger vessel with paraffin (*C*). The space between the two vessels was filled to within 2 cm. of the top with water (*D*) containing a little copper

sulfate and a few drops of sulfuric acid to prevent the growth of molds. The last 2 cm. were then filled with more paraffin (E) to prevent evaporation. The inner cylinder was next filled half full of a mixture consisting of 38 per cent bromobenzene and 62 per cent kerosene, having a resultant density of 1.06. Without disturbing the lower layer, an upper layer of specific gravity 1.00 (30 per cent bromobenzene, 70 per cent kerosene) was then carefully pipetted in until the level was 4 or 5 cm. below the top. The gradient was then produced by a few uniform strokes with a heavy copper wire (F) terminating in a flat coil (G) (as suggested by Dr. A. M.
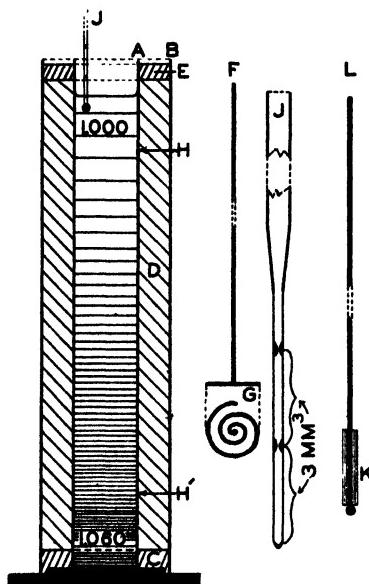
Fig. 2. Density gradient tube. A, 250 cc. graduated cylinder; B, 2 liter ungraduated cylinder; C, E, paraffin; D, dilute copper sulfate solution; F, stirring wire; G, spiral foot of F; H, H', beginning and end of gradient; J, J', pipette for delivery of two separate droplets into gradient tube; K, filter paper wrapped around L; L, a slender rod.

Butler). The strokes stopped 5 or 6 cm. from either end (H, H') to leave a reservoir of the lighter or heavier liquid. The gradient was then saturated with water by shaking 1 cc. of 15 to 20 per cent sodium chloride solution with 4 or 5 cc. of the lighter bromobenzene-kerosene mixture and then pouring this rapidly into the top of the gradient before the droplets had time to separate. After a few hours, the droplets had fallen through to the bottom, leaving the gradient saturated with water vapor.[2]

[2] K. Linderstrøm-Lang, personal communication.

For making the measurements, two droplets of about 3 c.mm. of each serum were dropped in by means of a pipette (J, J') only slightly different from that described by Linderstrøm-Lang and Lanz (14). At the same time, standard droplets of sodium chloride solutions were dropped in. Readings were made by noting the position, with respect to the graduations of the inner cylinder, of the upper or lower limb of a droplet 5 minutes after it had been put in. After a series of droplets had been read they were removed, as described by Linderstrøm-Lang, with a piece of moistened filter paper (K) wrapped around the end of a slender rod (L).

By interpolation of the readings of the standards, the density of the serum could be estimated with an accuracy of 0.00005. When a number of measurements were made at one time, it was convenient to plot the position of the standards against their density and then obtain the density of the unknowns directly from the resultant graph. The densities were used to calculate the protein contents according to the formula of Moore and Van Slyke (16), and subsequently the water content was calculated, assuming that, in addition to the protein, there were 15 gm. of solids per liter (assuming 9 gm. of salts, 4 gm. of lipid, 1 gm. of glucose, and 1 gm. of other extractives). An empirical expression for the calculation of the water content of the serum in gm. per liter in one operation from the serum density is water content at 20° = 1000 − 2350 (sp. gr. − 1.002). This embodies both the calculation of the serum protein from the formula of Moore and Van Slyke and the assumption of the presence of 15 gm. of non-protein solids per liter of serum. The gradient tube described will maintain a useful gradient for at least 6 months if kept away from radiators and direct sunlight.

Calculations

Calculation of Derived Data—After determination of the concentration of certain tissue constituents, it is possible to calculate various data of histochemical interest. These secondarily derived data furnish a histochemical description of tissue in terms of its intracellular and cellular moities. The subdivision of the tissue into histologically and chemically distinct compartments may be expressed by three symbols: the extracellular fluid, E; the total extracellular tissue, E_T; and the total cell weight, C. From the calculations of these major subdivisions of the tissue, it is possible to estimate the *concentration* of many substances in the two tissue compartments. Examples are the concentration of water in the *extracellular compartment*, $(H_2O)_E$, and the concentration of water, total phosphorus, and potassium in the *intracellular compartment*, $(H_2O)_c$, $(P)_c$, and $(K)_c$. These comprise the calculations of derived data to be presented and discussed in Papers II and III.

Partition of Tissue—All calculations have been made on a fat-free basis. In the case of *skeletal muscle*, the extracellular fluid weight, E, was calculated as described by Hastings and Eichelberger. This calculation is based on the assumptions that (*a*) chloride ion is confined to the extracellular space, (*b*) that it is present in the extracellular water at a concentration 5 per cent greater than in the serum water as a result of the Gibbs-Donnan effect, and (*c*) that the extracellular fluid contains 1 per cent solids, chiefly salts. That is,

$$E = \frac{\text{m.eq. Cl per kilo tissue}}{\text{m.eq. Cl per kilo serum water}} \times \frac{95}{99} \times 1000$$

The total extracellular weight, E_T, has been considered as equal to the weight of the extracellular fluid plus the collagen and elastin. The relative fiber weight per kilo of tissue, C, is then equal to $1000 - E_T$.

With the *heart* data, similar calculations were made except that the extracellular fluid, E, was considered to contain 5 per cent solids (1 per cent salt plus 4 per cent protein) in keeping with the observations of Drinker *et al.* (17) on the lymph from dog hearts. It has, therefore, been assumed that the Gibbs-Donnan ratio between serum and extracellular fluid would be 0.98 instead of 0.95. That is,

$$E = \frac{\text{m.eq. Cl per kilo tissue}}{\text{m.eq. Cl per kilo serum water}} \times \frac{98}{95} \times 1000 \quad \cdot$$

The heart values were placed on a blood-free, as well as fat-free, basis, assuming that the concentration of blood chloride equals 75 milliequivalents per kilo, the blood total phosphorus equals 18 mM per kilo, and the blood solids equal 200 gm. per kilo.

Concentrations in Tissue Compartments—Since the extracellular water is equal to 99 per cent of the extracellular fluid, the concentration of water in the extracellular compartment, $(H_2O)_E$, is equal to $(0.99\ E \times 1000)/E_T$. The concentration of water in the cells, $(H_2O)_C$, is then equal to $((\text{total tissue water} - 0.99 \times E) \times 1000)/C$. The concentration of total phosphorus in the cells, $(P)_C$, is equal to the concentration of total phosphorus of the tissue $\times 1000/C$, since, for the present purpose, there is but a negligible amount of extracellular phosphorus. In the case of potassium, the concentration in the cells, $(K)_C$, is calculated after a very small correction is made for the amount of potassium to be found in the extracellular fluid. For example, a certain muscle sample contained, per kilo of fat-free tissue, 109.3 milliequivalents of potassium and 844 gm. of fibers. Since a kilo of this tissue contained 149 gm. of extracellular fluid, or 0.149 kilo, there was $4 \times 0.149 = 0.6$ milliequivalent of extracellular potassium, assuming an average figure of 4 milliequivalents per kilo for the potassium

content of extracellular fluid. This left 109.3 − 0.6 = 108.7 milliequivalents of potassium in the intracellular compartment. This potassium was, therefore, present at an intracellular concentration, $(K)_c$, of $(108.7 \times 1000)/844$, or 129 milliequivalents per kilo of fibers.

SUMMARY

1. Analytical methods used in the histochemical study of age changes in certain tissues of the rat have been described. Detailed descriptions have been given of (a) the use of a nitric acid tissue extract for the determination of chloride and potassium; (b) the spectrophotometric estimation of hemoglobin in tissues in the presence of turbidity and myoglobin; and (c) the determination of serum density with the gradient tube.

2. Calculations are given, suitable for interpreting the analytical data in terms of the composition and amounts of intra- and extracellular phases.

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HISTOCHEMICAL CHANGES ASSOCIATED WITH AGING

II. SKELETAL AND CARDIAC MUSCLE IN THE RAT*

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The present inquiry is concerned with the quantitative histochemical changes which may occur with age in skeletal and cardiac muscle. As the result of the work of numerous recent investigators, it now seems possible to obtain significant morphological information from purely chemical measurements. If muscle may be considered as composed of two compartments, extracellular and intracellular, then the present problem may be formulated as an attempt to observe the quantitative alterations occurring with age in (*a*) the relative proportions of these extra- and intracellular compartments, (*b*) the composition of the extracellular compartment, and (*c*) the composition of the intracellular compartment.

The present study is limited to the determination of the relative proportions and water contents of the extra- and intracellular compartments in cardiac and skeletal muscle, the total phosphorus concentration in the cardiac and skeletal muscle fibers, and the concentration of potassium in the skeletal muscle fibers. The methods of analysis and calculation have been described in Paper I (1).

The theory has long been held that progressive desiccation of tissues occurs in old age. It has been implied that such desiccation resulted from a decrease in the water concentration of the intracellular tissue components (2). Tissue desiccation, if present, might also result from an increase in the proportion of tissue components relatively rich in solids. That a change in the proportion of extracellular and intracellular material can occur is implied by the analyses of Simms and Stolman on human autopsy material (3). These authors observed decreases in extreme age in the primarily intracellular substances, potassium, phosphorus, and solids, and increases in the chiefly extracellular elements, chloride and sodium.

The evidence to be presented in the present paper does not support the conclusion that senility in the rat, at least, is accompanied by tissue des-

iccation, but indicates that it may be accompanied by demonstrable changes in the proportion of extracellular and intracellular components of the tissue.

<div align="center">EXPERIMENTAL</div>

The animals used were rats of the Wistar and Yale strains. The Wistar rats, thirty-two in number, were bred in the Harvard laboratories; the Yale rats, 70 in number, were obtained from the Laboratory of Animal Nutrition of Cornell University.[1] The animals were sacrificed at different ages and their blood and tissues analyzed according to the methods described in Paper I.

The series of Wistar animals has been divided into five groups averaging 44, 70, 153, 449, and 668 days of age. If 10 days be considered the equivalent of a year in the life of man, then these groups are comparable to 4, 7, 15, 45, and 70 years of age in man. The Cornell series includes three groups of an average age of 60, 604, and 988 days. The 604 day-old group includes eleven rats, the tissues of which were analyzed individually, and forty-six rats from which the tissues were collected in four large pools for analysis. The age of the oldest Cornell group may be considered the equivalent of 100 years for man.

<div align="center">*Results*</div>

The analytical data resulting from the individual blood and tissue analyses will be presented in Tables I to III as the means of the different age groups and the standard deviation for each group. This method of presentation fails to reveal the fact that a change observed in any one tissue constituent of an individual animal was accompanied by a parallel change in other related constituents.

Serum—The serum was analyzed for protein and chloride in order to provide data required for the calculation of the amount of extracellular tissue fluid. These data for the different age groups are included in Table I. The changes of serum proteins with age reveal certain tendencies worth noting. As reported by Hatai (4) and others (5, 6), during growth serum protein concentration increases. As middle age is reached, the average serum protein levels rise still further, with only slight increases in the mean protein concentration thereafter. It is surprising that, in spite of the pathological changes in the kidney which are found almost constantly in older rats, few examples of definitely low serum protein concentrations were encountered. However, there appears to be more individual varia-

tion in the serum protein of the older than of the younger age groups. This tendency for a greater spread of analytical results in the aged animals was characteristic of most of the constituents studied.

Skeletal Muscle

In considering the histochemical results, there will first be presented the original analytical data, followed immediately by the physiologically more significant, derived data.

TABLE I

Mean Values for Serum and Skeletal Muscle; Original Data

The values are reported per kilo of serum or fat-free tissue.

	Age	No. of rats	Weight	Serum		Skeletal muscle					
				Cl	Pro-tein	H₂O	Col-lagen	Elastin	Cl	P	K
Wistar strain											
	days		*gm.*	*m eq.*	*gm.*	*gm.*	*gm.*	*gm.*	*m eq.*	*mM*	*m eq.*
	44	4	88	89.6	50	771	9.7		15.2	86.2	
σ				0.8	1	3	0.7		0.3	1.8	
	70	8	184	93.5	55	763	6.9		12.4	83.8	113.3
σ				2.5	3	3	0 8		1.6	1.0	4.0
	153	8	272	98.0	57	763	6.4	1.7	12.0	80.8	112.9
σ				2.5	1.5	2	0.9	0.4	2.2	1.2	2.0
	449	5	365	94.9	65	760	10.5	3 0	12.2	81.0	110.3
σ				2.5	4	3	4 6	0 3	0.5	7.6	4.3
	668	7	310	92.7	67	764	12.4		13 5	77.4	107.0
σ				3.4	7	7	4.3		1.7	2.7	2.0
Yale-Cornell strain											
	60	6	111	101.8	59	779	9.0	3 0	15.7	82.5	117.3
σ				3.6	4	7	2.5	1.0	2.0	0.9	5.5
	604*	57	311	100.3	64	762	10.8	1.9	12.7	76.3	110.6
σ†				3.1	4	6	3.6	1.1	1.4	2.2	7.8
	988	7	244	97.0	67	790	12.7	1.5	22.9	67.6	99.8
σ				3.5	6	16	4.9	0.6	5.8	6.9	9.9

* Consists of four large groups and eleven individual animals.
† Based on the single animals in the group.

Original Data—The changes observed in the skeletal muscle as a whole are not marked until extreme old age is reached (Table I). During *growth*, *i.e.* up to 153 days, there is a tendency for the concentration of water, collagen, chloride, total phosphorus, and potassium of the muscle to decrease, the greatest percentage change involving the chloride which decreases an average of 20 per cent. Yannet and Darrow (7) observed similar changes in the water and chloride concentration in the muscle of

cats during a comparable period of growth. They observed, however, an increase rather than a decrease in potassium and phosphorus during growth. This may be a species difference, since Cole and Koch (8) observed higher total phosphorus values in immature rats than in adults, in agreement with the present data.

During the *middle period* of the rat's life, from 153 to 668 days of age, there are but minor changes in the tissue constituents studied except for the collagen which tends to increase.

By the time the rats reach *old age*, at approximately 1000 days, rather marked changes occur. There is, on the average, a 10 to 12 per cent decrease in both the total phosphorus and the potassium. These decreases are accompanied by definite increases in the water and chloride concentration, the chloride increasing about 80 per cent. In the case of the muscle of the individual animals, the greater the decrease in potassium and phosphorus, the greater the increase in water and chloride concentration. A greater variability in the values for each tissue component is also evident in the oldest group as compared to the younger groups, some of the values approaching the normal averages for a younger age, while others are very markedly altered. In general, the changes observed in the rat muscle are similar to those found by Simms and Stolman in human material (3). It may be stressed that during senescence, an actual increase in the water concentration of muscle occurred instead of the decrease which had been anticipated.

DISCUSSION

Skeletal Muscle

Derived Data—From the original data just presented, certain data of morphological interest may be derived as detailed in Paper I (1). These *histochemical* data (Table II) serve to describe the changes occurring during aging in terms of the events taking place in the fibers themselves, and in the amount and nature of the surrounding extracellular tissue, rather than in terms of the muscle as a whole.

The *extracellular compartment*, E_T, is the sum of the extracellular fluid, E, and the collagen and elastin. The individual values for E_T of muscle from 44 to 1000 days of age are shown in the upper portion of Fig. 1. During the period of growth, *i.e.* from 44 to 153 days, there appears to be a 25 or 30 per cent decrease in the relative mass of the extracellular compartment. Subsequent to the period of growth, there is little further change in E_T until the rats are over 600 days of age. In the oldest group at 988 days of age, the average value for the extracellular compartment was 222 gm. per kilo of muscle, an increase of about 80 per cent over the average for the younger adult groups. It will also be noted that the variation from individual to individual in the oldest age group increases markedly.

The composition of the extracellular compartment would not appear to undergo alterations in composition of significant magnitude during the life of the rat. It may be noted that, contrary to expectation, the water concentration of the extracellular compartment $(H_2O)_E$ actually increased somewhat in the oldest age group instead of decreasing.

The changes in the relative amount of extracellular compartment may not be without important functional consequence to the metabolism of the tissue cells. Conceivably, an increase in the amount of interstitial fluid

TABLE II

Mean Values for Skeletal Muscle; Derived Data

E, E_T, and C are reported per kilo of fat-free tissue; $(H_2O)_E$ per kilo of E_T; $(H_2O)_C$, $(P)_C$, and $(K)_C$ per kilo of C.

	Age	No. of rats	Weight	E	E_T	C	$(H_2O)_E$	$(H_2O)_C$	$(P)_C$	$(K)_C$
	Wistar strain									
	days		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mM*	*m.eq.*
	44	4	88	152	163	837	925	740	103	
σ				3	3	3	4	5	2	
	70	8	184	116	124	876	923	740	96	130
σ				17	16	16	7	5	2	4
	153	8	272	109	117	883	920	742	91	128
σ				19	19	19	11	7	3	4
	449	5	365	113	128	872	893	740	93	126
σ				7	7	7	26	7	9	4
	673	6	320	128	143	857	895	741	90	125
σ				17	14	14	24	6	2	5
	Yale-Cornell strain									
	60	6	111	139	150	850	913	756	97	138
σ				14	15	15	20	9	2	5
	604	57	311	112	125	875	889	742	87	126
σ				13	16	16	17	7	3	8
	988	7	244	208	222	778	927	752	87	128
σ				55	62	62	9	7	3	7

would increase the average distance between capillaries and fibers and, thereby, decrease the rate of interchange of metabolites.

An increase in the amount of extracellular compartment signifies a corresponding decrease in the relative mass of the *intracellular compartment, C.* The 12 per cent decrease in this portion of the tissue which occurs between the ages of 153 and 988 days has interesting implications. Since the muscles constitute the largest soft tissue mass of the body, the total active cytoplasm in an old rat is apparently much less than is anticipated from the body weight alone. The basal metabolic rate is a case in point. Even

though the cytoplasm retained the same resting metabolism in the old rat as in the young, the total metabolism per unit of body weight would appear to be lower, because a large proportion of the body weight would now consist of inactive extracellular material.   It is possible that the decrease in basal metabolism observed in older people might be explained on this basis.

The changes observed in the proportion of the extracellular compartment with growth and with aging may result from similar causes operating in opposite directions.   Yannet and Darrow (7) point out that, as the developing muscle fibers enlarge, they might be expected to pack more tightly, leaving less room for extracellular fluid than in the more loosely constructed younger muscle.   The muscles of very old rats are much reduced in size. This is not surprising, since one might anticipate rather extensive atrophy
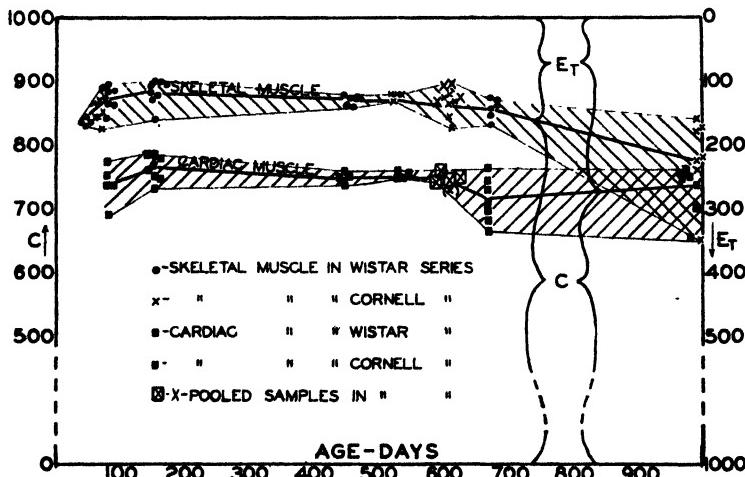
FIG. 1. Changes in the proportions of extracellular and intracellular values in muscles of rats during aging.

of disuse as a result of the inactivity of older animals.   Superficially, the atrophy of muscle fibers could easily lead to a morphological condition not unlike that in the young animal, inasmuch as the fibers would be smaller and perhaps less tightly packed.   Hines and Knowlton found chemical changes in experimental muscular atrophy which were similar to those observed in senility (9).   There was a greatly increased extracellular compartment.   It remains to be seen whether it is atrophy or an actual loss of fibers which accounts for the decrease in the size of the muscle of very old rats.

As for the *changes in the intracellular compartment* proper, it may be said in general that a few changes are observed during growth, but that subsequently the muscle fibers, as far as the present data go, appear to remain

very nearly constant in their chemical composition. During the period of growth, after 44 days of age, there appears to be a significant decrease in the concentration of both phosphorus and potassium in the fibers. This is not meant to imply that the total base concentrations in the fibers actually diminish during growth, since the changes in potassium and phosphorus might be compensated by alterations in other intracellular cations and anions. In the oldest age group, there is no evidence of change in the intracellular concentration of potassium or phosphorus.

Of greater interest is the observation that *the water concentration in the fibers* $(H_2O)_c$ remains relatively constant throughout life. The constancy is best seen in rats of the Wistar series. The youngest and oldest Cornell
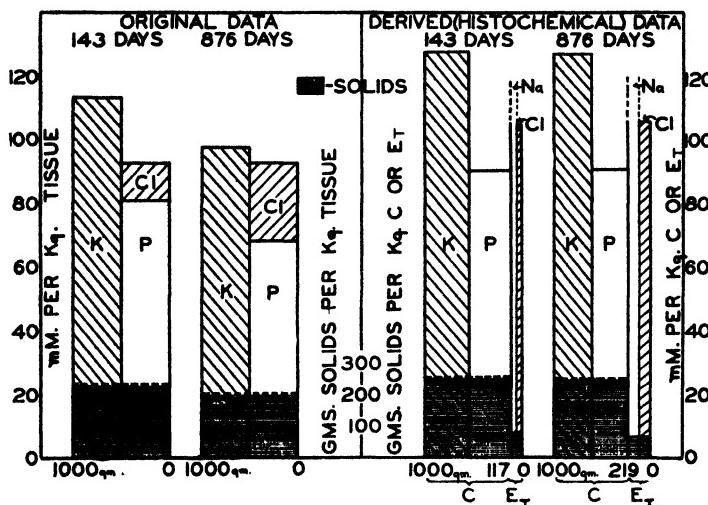
FIG. 2. Histochemical changes in muscle during aging, as shown by a comparison of data from skeletal muscle of two rats, one 143 and the other 876 days of age.

groups have slightly higher average water concentrations than the rest, but it is not certain whether these represent real differences. In any event, the changes are minor when compared to the large differences in tissue water found in the original data. This brings to the fore the interesting question of whether there will be found any actual difference in the composition of individual functioning muscle cells in old age as compared with those of the mature animal; and whether the change in physiological function of a tissue may not rest in the change in the relation between the cell and its environment.

In order to emphasize the nature of the histochemical changes in the muscle in old age, there are shown in Fig. 2 diagrams of the data from the skeletal muscle of two individual rats, one 143 and the other 876 days of age.

At the left, the original data for phosphorus, potassium, chloride, and solids are represented as though they were evenly distributed throughout the tissue. The total solids are superimposed over the bottom of both columns. In the older animal, the higher value for chloride and the lower values for potassium, phosphorus, and solids are evident. At the right side of the chart, the distance along the abscissa has been divided into segments representing the extra- and intracellular compartments of the tissue. Each of these segments is again divided in two, corresponding to the cations and anions which are represented by potassium and phosphorus in the intracellular portion, and by sodium and chloride in the extracellular portion, each of these appearing at the concentration at which it occurs in its respective compartment. (Sodium was not determined in these experiments, but its presence is indicated simply to provide a covering cation for the chloride anion.) These diagrams should not be regarded as complete ionic patterns of muscle, but only graphical representations of the data obtained in the present investigation. Thus represented, the intracellular concentrations of potassium, phosphorus, and solids appear to differ but little in the old and young muscle.

This is an illustration of how the changes in chemical composition of whole muscle occurring during senescence may, by histochemical interpretation, reveal an increase in the proportion of the extracellular compartment of muscle, a corresponding decrease in its intracellular compartment, and the lack of significant change in the composition of either the extra- or intracellular compartments.

Cardiac Muscle

The *original data* for the cardiac muscle of rats of different ages, consisting of water, collagen, elastin, chloride, and phosphorus determinations, are given in Table III. (Potassium determinations were not included in this series of analyses.) The data were obtained on four age groups of Wistar strain animals and two advanced age groups of the Yale-Cornell strain. Although the data are insufficient to show the trends with growth, slight increases in chloride and decreases in total phosphorus occur in the oldest age group of each strain. In contrast with skeletal muscle, there is no increase in collagen in the cardiac muscle of the aged rats.

By reference to the derived data, also included in Table III, one may better visualize the histochemical meaning of the changes in constituents studied. In general, it would appear that the histochemical changes in the heart with aging are slight. In confirmation of the work of others (10, 11), the extracellular compartment of cardiac muscle, E_T, is about twice that of skeletal muscle in young and adult rats. In the oldest age group (988 days), the difference is not so marked, although there appears to be some

increase in the extracellular compartment of heart muscle in this group. This means a corresponding decrease in the intracellular compartment. These changes in E_T and C have been plotted in the lower part of Fig. 1. As in the case of skeletal muscle, the spread of the individual values is greatest in the oldest age group.

The concentration of water in the two phases of the cardiac tissue shows no significant change with advancing age. Nor would there appear to be any evidence that the concentration of intracellular phosphorus of the heart

TABLE III

Mean Values for Cardiac Muscle

The original data, E, E_T, and C are reported per kilo of blood-free, fat-free tissue; $(H_2O)_E$ per kilo of E_T; $(H_2O)_C$, and $(P)_C$ per kilo of C.

	Age	No of rats	Weight	H₂O	Col-lagen	Elas-tin	Cl	P	E	E_T	C	$(H_2O)_E$	$(H_2O)_C$	$(P)_C$
					Original data						Derived data			
	days		*gm.*	*gm.*	*gm.*	*gm*	*m.eq.*	*mM*	*gm*	*gm*	*gm*	*gm.*	*gm.*	*mM*
						Wistar strain								
	71	6	184	772	6.7		24.8	87.6	253	260	740	924	721	118
σ				8	1.8		2.6	1.9	26	27	27	5	5	5
	153	8	272	778	5.0	1.2	23.4	82 8	227	236	764	925	732	108
σ				7	2.2	0.7	1.3	2.1	16	16	16	7	8	5
	449	5	365	777	6.9	2.8	24.2	80.4	242	251	749	915	730	107
σ				5	1.3	0.2	0.5	4.0	7	7	7	5	7	5
	668	7	310	779	7.0		27.0	77.4	277	286	714	919	724	106
σ				7	1.4		2.6	3.0	33	33	33	5	6	11
						Yale-Cornell strain								
	604	47	332	780	8.3	1.2	25.1	77.9	239	249	751	914	734	103
σ*				2	1.3	0.1	0.4	1.7	4	4	4	5	4	5
	988	7	244	785	9.0	2.1	26.6	71.8	260	272	728	911	739	99
σ				7	3.2	0.9	3.2	2.0	35	36	36	16	7	6

* Estimated from the results for four individuals.

changes as the result of aging of the animal. These observations may be regarded as further evidence for the thesis that in old age the chemical changes found in a tissue reflect a change in the proportion of the different morphological components of the tissue rather than a change in the composition of the individual component parts.

SUMMARY

1. The concentrations of water and chloride in the skeletal muscle of the rat decrease during growth and rise again in old age. The concentrations

of potassium and phosphorus likewise decrease during growth but continue to fall in senescence. The changes in cardiac muscle are similar but much less marked. Considerable variation exists in the extent of the chemical changes in the muscle of the oldest animals.

2. When these changes are interpreted histochemically, there appears to be an increase during growth in the proportion of intracellular tissue at the expense of the extracellular compartment. In the middle period of life, little change is observed; but in senescence, the relative mass of the intracellular compartment decreases, particularly in skeletal muscle, leaving a greatly increased extracellular compartment. In the adult, the proportion of extracellular material is much greater in cardiac than in skeletal muscle and the increase with age is much less extensive.

These changes in the relative amounts of the major tissue compartments may have important consequences: (a) the increase in the amount of extracellular fluid in skeletal muscle in old age may affect the efficiency of exchange between blood and fibers; (b) the variation during life in the proportion of fibers in a given weight of muscle may explain some of the changes occurring in the metabolic activity and mechanical ability of muscle at different ages.

3. During senescence, no evidence was found for desiccation of either the whole muscle, the extracellular portion of tissue, or the fibers.

4. The changes in the water and solids of the extracellular compartment of cardiac and skeletal muscle during aging were slight. The concentration of collagen in the extracellular compartment was not increased in old age.

The intracellular concentration of phosphorus and potassium decreased during growth in skeletal muscle; but during senescence, the concentration of water, total phosphorus, and potassium in the muscle fibers underwent no significant change. Similarly, senescence produced no definite change in the water and total phosphorus concentration in the cardiac muscle fibers.

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HISTOCHEMICAL CHANGES ASSOCIATED WITH AGING

III. THE EFFECTS OF RETARDATION OF GROWTH ON SKELETAL MUSCLE*

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It has been shown that by retarding the growth of the white rat, the life span may be considerably extended (McCay and Crowell (1)). Such retarded animals retain an appearance of youth at a time in life when well fed animals are showing signs of advanced age.

The question arises as to whether significant histochemical differences exist between the tissues of these small long-lived rats and those of the much larger control animals whose life expectancy is far less. In Paper II there were described characteristic histochemical changes in skeletal muscle during growth and senescence in the rat (2). In this paper, data on the skeletal muscle in the retarded animal will be compared with those of the unretarded animal. The results obtained suggest that the muscles of 2 year-old retarded animals resemble those of growing, rather than mature or senescent animals.

EXPERIMENTAL

The rats used were all reared at the Laboratory of Animal Nutrition of Cornell University, and were of the Yale strain. Samples of skeletal muscle were obtained from a total of thirty-one animals belonging to four groups. There were two groups of retarded animals averaging 611 and 752 days of age, a control group of well fed animals at 610 days of age, and finally a group of 60 day-old immature rats of approximately the size of the retarded animals. The age of these groups at 60, 600, and 750 days would be comparable to an age for man of 6, 60, and 75 years.

The retarded groups were maintained on a diet adequate except for caloric intake, and they were about one-third of the normal size, having been permitted to gain only 5 gm. per month after weaning. The 610 day-old control group received the same basal mineral and vitamin diet as the

retarded animals plus added supplements of fat, carbohydrate, and protein. Complete descriptions of the diet, autopsy findings, and life expectancy of the retarded rats have been previously reported (3, 4).[1]

The analytical methods and histochemical treatment of the data have been given in Paper I (5).

Results

The analytical and derived data which have been obtained are shown in Table I. These data are given as the average for each group, together with the standard deviation within the group.

Original Data—The concentrations in the muscle of water, collagen, elastin, chloride, total phosphorus, and potassium are recorded. The two retarded groups at 611 and 752 days of age are similar to each other in respect to the original analytical data. Both retarded groups differ from the full grown 610 day-old control animals in showing higher muscle concentrations of water, chloride, phosphorus, and potassium. The high values for these four substances in the muscles of the retarded animals approach the concentrations found in the muscle of young animals of comparable size, but of only one-tenth the age.

No significant differences are found in the collagen and elastin values for the different groups. Retardation apparently does not result in an increase in fibrous tissue in the muscle.

Derived Data—These original data have been interpreted histochemically in the manner described in Paper I (5). The resultant derived data are recorded in Table I as (*a*) the gm. of extracellular material, E_T, and intracellular material, C, per kilo of fat-free tissue, (*b*) the water concentration in the extracellular compartment, $(H_2O)_E$, and (*c*) the water, total phosphorus, and potassium concentration in the muscle fibers, $(H_2O)_C$, $(P)_C$, and $(K)_C$.

In general, the muscle of the retarded animals appears to resemble that of the animals of similar *size* rather than that of the non-retarded animals of similar *age*. In comparison with the muscle of full grown animals of 610 days of age, there is a greater proportion of extracellular tissue in the muscle of the retarded groups, the difference amounting to 12 to 20 per cent. This is equivalent to the presence of 2 to 4 per cent less intracellular tissue in a given mass of the retarded muscles. There is no significant difference between the water concentration in the extracellular muscle compartment of the retarded rats and of the well fed age controls. The intracellular water concentration is not significantly higher in the retarded groups than in the

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[1] The animals used were those from the third study of retarded growth made at Cornell. A complete description of the experiment will be published on its termination in about 1943.

unretarded, but the concentrations of both potassium and total phosphorus are definitely greater. The values for potassium and phosphorus lie intermediate between the high concentrations in the muscle fibers of the young animals and the low concentrations in the unretarded 610 day-old controls.

In Paper II (2), it was found that during growth the proportion of extracellular material in muscle decreased, and that the intracellular concentration of potassium and phosphorus also fell. These changes took place during the first 150 days of the life of the rat. Thus, the muscle of the retarded 600 and 750 day-old animals seems to resemble the muscle of immature 60 day-old rats more closely than that of either young or middle-aged adults. It is a temptation to conclude that the retardation in the growth of the rat muscle has resulted in the persistence of muscle fibers in

TABLE I

Effect of Retardation on Skeletal Muscle

The original data, E_T, and C are reported per kilo of fat-free tissue; $(H_2O)_E$ per kilo of E_T; $(H_2O)_C$, $(P)_C$, and $(K)_C$ per kilo of C.

	Original data									Derived data					
	Age	No. of rats	Weight	H_2O	Collagen	Elastin	Cl	P	K	E_T	C	$(H_2O)_E$	$(H_2O)_C$	$(P)_C$	$(K)_C$
	days		gm.	gm.	gm	gm	m eq	m M	m eq	gm	gm.	gm.	gm.	mM	m.eq.
Size control	60	6	111	779	9.0	3.0	15.7	82.5	117.3	150	850	913	756	97	138
σ			7	2.5	1.0	2.0	0.9	5.5		15	15	20	9	2	5
Age control	610	7	328	759	10.1	2.5	13.6	74.5	110.1	140	860	899	738	88	128
σ			7	3.0	1.2	1.7	2 1	5.1		18	18	15	7	3	6
Retarded	611	8	104	771	10.9	2.6	17.3	76.6	113.4	169	831	910	746	92	137
σ			7	2.1	1.3	1.4	1.5	3.3		12	12	13	8	2	7
"	752	10	121	770	11.4	3.5	16.9	79.7	112.7	157	843	897	747	95	134
σ			6	3.9	0.8	2.6	2.1	2.7		23	23	18	8	2	4

their youthful state. It will be necessary, however, to wait for fuller evidence, both chemical and functional, before concluding that a 750 day-old retarded animal has muscles resembling those of an animal one-twelfth as old.

SUMMARY

1. In 611 and 752 day-old rats whose growth had been retarded through the limitation of caloric intake, the skeletal muscles contained more chloride, water, total phosphorus, and potassium than those of well fed controls of 610 days of age.

2. These differences have been interpreted histochemically as signifying a higher proportion of extracellular tissue, and higher intracellular concentrations of potassium and total phosphorus in the retarded animals.

3. These findings are compatible with the hypothesis that retardation results in the maintenance in muscle of youthful muscle fibers and a youthful proportion of fibers and extracellular tissue.

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THE EFFECT OF PROGRESSIVE IODINATION ON THE THYROIDAL ACTIVITY OF IODINATED CASEIN*

By E. P. REINEKE, M. B. WILLIAMSON, AND C. W. TURNER

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During recent years, the fact that substances with thyroidal activity can be formed by the simple iodination of proteins has become well established. Abelin and Florin (1) and Abelin (2) reported the recovery of highly active acid-insoluble fractions from barium hydroxide hydrolysates of iodo-proteins. Abelin and Neftel (3) demonstrated that both the type of protein and the iodination method used are important factors in determining the activity of the final product. Hydrolytic products of iodinated native proteins showed marked activity. Direct iodination of peptone and ereptone, on the other hand, failed to yield substances with thyroidal activity. Iodination in bicarbonate yielded better results than in ammonia solution.

Ludwig and von Mutzenbecher (4) confirmed by Harington and Rivers (5) succeeded in isolating thyroxine from iodinated casein after hydrolysis with barium hydroxide.

Lerman and Salter (6) reported the correction of myxedema in human patients and in thyroidectomized rabbits by the administration of iodinated serum protein and its acid-insoluble degradation products. In our own laboratory the administration of iodinated casein in graded doses to young thyroidectomized goats arrested the development of cretinism and stimulated growth in proportion to the amount of material given (Reineke and Turner (7)).

Earlier work (unpublished) indicated that the iodination of casein or skim milk by a variety of procedures led to large differences in activity, apparently due to variations in several factors or combinations of factors. This suggested the desirability of establishing more definitely the conditions influencing the reaction whereby substances with thyroidal activity are produced.

Iodination Procedure

Two different series of iodinated proteins were prepared in order to determine the effect of progressively increasing iodine on their thyroidal activity.

Series I was prepared by the direct iodination of skim milk. 700 ml. of skim milk were placed in a glass jar, 5 gm. of sodium bicarbonate were added, and the container was placed in a water bath maintained at 38–40°. The mixture was agitated vigorously and continuously by means of a glass stirring rod with a triangular foot, attached to a motor stirrer. Finely powdered iodine was then added at intervals, and in small amounts, over a period of 3 to 4 hours. When the required amount of iodine had been added, the jar was closed with a rubber stopper having a glass bearing to accommodate the stirrer. The closed container and stirring apparatus were placed in an incubator at 38°, with continuous stirring, for 18 to 20 hours. pH readings were taken with a glass electrode pH meter after addition of the bicarbonate, after addition of all the iodine, and after the incubation period. The iodinated protein was recovered by careful addition of dilute HCl until the point of maximum precipitation was reached (pH 3.8 to 4.0). After several washings with water adjusted to pH 4.0, the greater part of the iodinated protein was removed by filtration, dried at room temperature, ground in a laboratory mill, and assayed without further treatment. A small portion from each member of the series was resuspended with a minimum amount of NaOH and dialyzed across a cellophane casing in order to eliminate loosely combined iodine. The iodoprotein was then precipitated by the addition of HCl, filtered, and dried. Iodine analyses were made on both the dialyzed and the undialyzed portions by the method of Kendall, as described by Harington (8).

Series II was made up with casein that had been prepared in the laboratory from fresh, unpasteurized milk. 21 gm. of casein were placed in 700 ml. of distilled water to which 5 gm. of sodium bicarbonate had previously been added, with vigorous stirring until the casein dissolved. The remainder of the process was then carried out exactly as in Series I.

Methods of Assay

The method of Kreitmair (9), based upon the weight loss of guinea pigs, has been used extensively for the assay of thyroid-active materials. Dressler and Holling (10) have published a method of assay based upon the increase in oxygen consumption of guinea pigs stimulated by a given dose of material.

The fact that thyroid material will stimulate precocious metamorphosis in frog tadpoles (Gudernatsch (11)) has been used widely both as a qualita-

tive and a quantitative measure of thyroid activity. Gaddum (12) reported that when tadpoles were exposed to thyroxine for 24 to 48 hours the decrease in body length bore a rough quantitative relationship to the amount of substance employed. Wokes (13) devised a method for the quantitative assay of thyroid substance on tadpoles.

Assays on Guinea Pigs—For the assay of the iodinated proteins reported in this paper, modifications of the three methods noted above were employed. Both the increase in oxygen consumption and the percentage decrease in weight loss of guinea pigs were determined on the same animals. Healthy male guinea pigs weighing from 230 to 280 gm., averaging approximately 250 gm., were used. In order to have the animals in a partially fasted state for daily measurements of metabolism, all food was removed from the pens at night and the animals were allowed access to food only during the day, after the oxygen consumption had been recorded. This plan of treatment was used during the experimental period and also during the week preceding it. Control animals handled similarly gained an average of 2 per cent per week. The diet consisted of 80 per cent of a grain mixture and 20 per cent Cerogras,[1] added as a vitamin supplement.

In preparation for administration, a weighed amount of the iodinated protein was put in solution in distilled water by addition of 2 or 3 drops of saturated sodium carbonate solution and triturated with a mortar and pestle. The dissolved material was then made up to a given volume and accurately measured quantities were given orally once daily for 6 days to guinea pigs by the method of Pugh and Tandy (14).

On the 4th and 5th day after beginning the dosage, measurements of oxygen consumption[2] were made in an eight chamber respiration apparatus of the same construction as that described by Winchester (15), but designed to accommodate animals between 150 and 300 gm. in weight. The percentage increase in oxygen consumption was calculated from the increase above normal controls of the same weight and maintained under the same conditions. Dosage of the animals was continued for 6 days and the final weights were taken on the 7th day after treatment was begun. Weight decreases were expressed as the per cent difference between the initial weight and the final weight of the animals.

Assays on Tadpoles—In contradistinction to the results obtained with thyroxine or thyroid substances it was found that tadpoles will give little or no response when placed in a solution or suspension of iodinated casein.

However, when the material was placed on the surface of the water as a fine powder so that the tadpoles could eat it, or when injected into the body cavity, the response was qualitatively indistinguishable from that of thyroid or thyroxine.

For the assays reported herein large frog tadpoles (*Rana pipiens*) of about 60 mm. in length were obtained by seining from a local pond. At this stage the rear legs were fully formed, but still non-functional, and the front limb buds had not yet emerged. Such tadpoles are extremely sensitive to stimulation and furthermore can be injected quite readily. Since the response obtained with tadpoles will vary widely from time to time, depending upon their stage of development, environmental temperature, and possibly other factors, all tadpoles to be used for the assay of a given series of preparations were injected on the same day. Final measurements of body length were made on the 4th day after injection. The same amount of iodoprotein from each member of the series was injected into the tadpoles of its respective test group. Therefore, the per cent decrease in body length provides an index of the relative potencies of the various members of the series.

Results

Data on the progressive iodination of skim milk and casein are presented in Table I. In Series I skim milk was iodinated directly by addition of iodine in concentrations ranging from 3.9 to 31.5 gm. per 100 gm. of protein. For purposes of calculation, the protein content of the milk used is assumed to be 3.5 per cent. While this assumption will introduce some error, it is believed to approximate closely the actual protein content of the mixed milk used.

Analyses of the iodoproteins before and after dialysis indicate that from 1 to 2 per cent of the iodine is in loose combination with the protein. This iodine can also be liberated by brief oxidation with hydrogen peroxide, potassium persulfate, or potassium permanganate in acid solution. After dialysis, however, no evidence of free iodine could be obtained after oxidation with the above reagents. Thus it is evident that the non-dialyzable iodine is in firm combination, presumably with the tyrosine of the protein. It is well established that the iodination of tyrosine proceeds by substitution according to the equation, tyrosine $+ 2I_2 \rightarrow$ diiodotyrosine $+ 2HI$. In the present series somewhat less than one-half of the iodine added remained in firm combination with the protein after dialysis. Since some side reactions doubtless take place in the mixed systems used, this is in good agreement with the theoretical expectation.

In Series II more accurate computations are possible, because casein of known tyrosine composition was used throughout, and the intervals be-

tween amounts of iodine added were spaced more closely than in Series I. It is of interest to note that until 4 atoms of iodine were added or 2 atoms combined per mole of tyrosine, the amount of iodine remaining in combination after dialysis was close to the theoretical value. There was then a lag in the combination of additional iodine (dialyzed values) until excessive amounts were added. However, the total amount of iodine combined increased progressively with increasing addition of iodine.

TABLE I

Course of Progressive Iodination of Skim Milk Proteins and Casein

Series No.	Preparation No.	Iodine added per liter milk	Iodine* added per 100 gm. protein	Iodine† added per mole tyrosine	Per cent iodine combined		Iodine combined per mole tyrosine (after dialysis)	pH		
					Before dialysis	After dialysis		After bicarbonate addition	After iodine addition	After incubation
		gm.	gm.	atoms			atoms			
I. Direct iodination of skim milk	1	1.428	3.939	1.24	8.47	2.20	0.72	7.20	7.01	7.62
	2	2.857	7.878	2.47	5 34	4.23	1.41	7.21	7.23	7.23
	3	4.285	11.817	3.71	6.95	5.13	1.73	7.45	7.61	7.60
	4	5.714	15.756	4 95	8.08	6.21	2.11	7.47	7.21	7.03
	5	7.142	19.695	6.18	8.48	6.97	2.39	7.31	7.02	7.25
	6	8.571	23.634	7.42	9.57	7.57	2.62	7.54	6.89	6.81
	7	10.000	27.573	8.66	10.00	7.95	2.76	7.57		6.65
	8	11.428	31.512	9.89	10.41	8.33	2.90	7.63		6.33
II. Iodination of casein	1		7.904	2.00	4.12	3.77	0.99	7.47	7.51	7.41
	2		11.904	3.00	6.10	5 17	1.38	7.47	7.09	7.07
	3		13.857	3.50	7.35	6.02	1.62	7.61	7.48	7.98
	4		15.809	4.00	8.62	6.80	1.84	7.61	7.10	7.21
	5		17.619	4.45	9.16	7.22	1.97	7 78	7.28	7.42
	6		20.000	5.05	9.56	7.51	2 05	7.78	7.05	7.85
	7		21.904	5.53	10.38	7.50	2.05	7.20	7.30	7.65
	8		24.285	6.14	12.25	8.78	2.43	7.20	7.13	7.19

* Skim milk was assumed to contain 3.5 per cent protein.

† Calculated on the basis of 4.5 per cent tyrosine in the mixed proteins of skim milk and 5.65 per cent tyrosine in casein. Tyrosine was determined by the method of Lugg (16).

In both series the pH of the reaction mixtures remained fairly close to the physiological range throughout. The final low values of 6.3 to 6.8 in Series I are probably due to depletion of the buffer capacity of the solutions during excessive iodination by the HI formed as a side product in the reaction.

Thyroidal Activity—Results of the assay of preparations in Series I by three different methods and of Series II by the tadpole method are given

in Fig. 1. The test animals for each assay in a given series were dosed with the same amount of material. Therefore, the actual per cent response gives a comparative measure of the potency of the respective preparations in a series.

Thyroidal activity increased with increasing iodine concentration, attaining a maximum when 4.5 to 5.0 atoms of iodine had been added per mole of tyrosine in the protein, an amount slightly in excess of that theo-
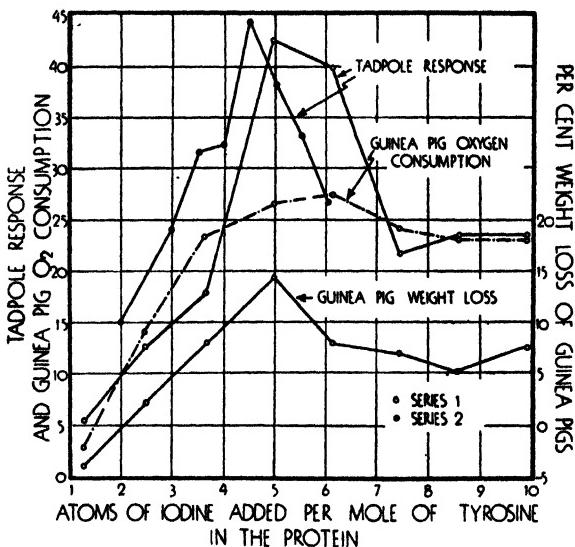
FIG. 1. The effect of progressive iodination on the thyroidal activity of casein and skim milk proteins. The results are expressed in terms of the amount of iodine added to the reaction mixture; this is somewhat more than twice the amount actually combined (see Table I). The weight losses for guinea pigs are based on eight animals, and metabolic results on four animals per assay. Iodinated protein was administered orally at the rate of 6 mg. daily per animal. The tadpole assays include five animals per preparation in Series I, and four per preparation in Series II. Each tadpole was given a single injection of 0.1 mg. of iodinated protein.

retically required for substitution of 2 atoms of iodine on the tyrosine ring. A striking feature of these results is that further iodination led to marked loss of activity.

In the assays of Series I, results of the tadpole test and the weight loss of guinea pigs are nearly parallel throughout the series, the only difference being one of degree of response. A second test run on groups of twenty artificially reared tadpoles given the preparations orally was in good agreement with the one illustrated. The t values were computed as described by Snedecor (17) in order to test the significance of the difference between

each member of the series and the preceding preparation. Results by the method of guinea pig loss in weight and the tadpole test revealed that both the rise in activity with progressive iodination and the decline after the maximum are statistically significant.

The increase in oxygen consumption of guinea pigs follows the same general trend as is shown by the other two methods. In this particular instance, however, the results are considered to be less quantitative than those obtained by the other methods, because it was observed during the course of the assays that the normal metabolism declined with the change from spring to summer conditions. This made it difficult to establish an accurate normal base from which to calculate the increase in oxygen consumption of the test animals. With this limitation in mind, it is believed that the metabolic data afford valuable confirmation of the results obtained by the other methods.

Results of the tadpole assay on the preparations of Series II are in good agreement with those of Series I, showing again an increase in activity up to a maximum when 4.5 atoms of iodine had been added per mole of tyrosine, followed by a decline in activity with further iodination. The results of this assay were confirmed by a second trial in which alternate members of the series were tested by injection into groups of twenty tadpoles. In both trials, computation of the statistical t values for the differences of response in succeeding members of the series indicated that both the rise in activity and the decline from the maximum are significant.

As judged by both the metabolic response and the weight loss of guinea pigs, preparations iodinated under optimal conditions show a potency of 0.01 to 0.005 of that of thyroxine when both materials are given orally. These results compare favorably with the activity of 1/300 that of thyroxine reported by Harington and Rivers (5) for iodinated casein. u.s.p. desiccated thyroid tested in .the same way showed about 0.005 of the activity of thyroxine. Lerman and Salter (6) reported that iodinated serum albumin had a potency of about one-fifth that of thyroglobulin.

<div align="center">DISCUSSION</div>

From the results, it is apparent that the degree of iodination of a protein is a controlling factor in the amount of thyroidal activity attained. Under the conditions of these experiments, thyroidal activity reaches a maximum when sufficient iodine has been added to substitute 2 atoms on the tyrosine ring. Further iodination leads to significant losses of activity. This is in agreement with the report (4) that excessively iodinated proteins fail to yield thyroxine after hydrolysis.

The iodination of serum albumin in a water-alcohol-ammonia medium by addition of compound solution of iodine has been reported by Muus,

Coons, and Salter (18). According to these authors thyroidal activity did not begin until 2 to 3 atoms of iodine per molecule of tyrosine were combined. Full activity was obtained only when 9 to 10 per cent of iodine or the equivalent of 4 atoms per molecule of tyrosine had been incorporated. No decrease of activity was noted with excessive iodination. The optimally iodinated preparations contained more iodine than could be accounted for by substitution on both the tyrosine and histidine of the protein.

As shown by the data in Table I, the iodination method used in the present work favors the substitution of iodine on the tyrosine ring, with little or no substitution in other parts of the protein molecule. Thus the marked differences in the course of formation of the thyroidally active product can probably be explained by the differences in the methods used.

The formation of thyroxine by the iodination of proteins could be accounted for (4, 5) by (a) the oxidative coupling of 2 molecules of diiodotyrosine with the elimination of one side chain or (b) the iodination of preformed thyronine which may exist as part of the protein molecule. The difficulties in the way of acceptance of both possibilities are pointed out by Harington and Rivers (5). In favor of the first mechanism is the fact that thyroxine has been formed directly from diiodotyrosine by prolonged incubation in alkaline medium (von Mutzenbecher (19), Block (20)). The increase in thyroidal activity with progressive iodination as reported herein is compatible with the first hypothesis. The loss of activity as excessive amounts of iodine are added could be explained by the coupling of a 3rd tyrosine molecule, forming an inactive compound such as the thyroxine analogue reported by Bovarnick et al. (21). As an alternative explanation, it appears possible that prolonged oxidation could result in changes in the remaining side chain of the thyroxine formed in the initial stages, resulting in degradation products with reduced activity.

SUMMARY

1. Casein and total skim milk proteins, buffered with sodium bicarbonate, were combined with progressively increasing amounts of iodine, and the iodine content and thyroidal activity of the resulting iodoproteins were tested.

2. It was found that under these conditions of iodination thyroidal activity reaches a maximum when sufficient iodine has been combined to substitute 2 atoms of iodine on the tyrosine ring. Further iodination results in a significant decrease in thyroidal activity.

3. From a consideration of the iodine contents of the dialyzed iodoproteins in relation to their original tyrosine content, it appears that the method of iodination used favors substitution on the tyrosine ring, with little or no substitution in other parts of the protein molecule.

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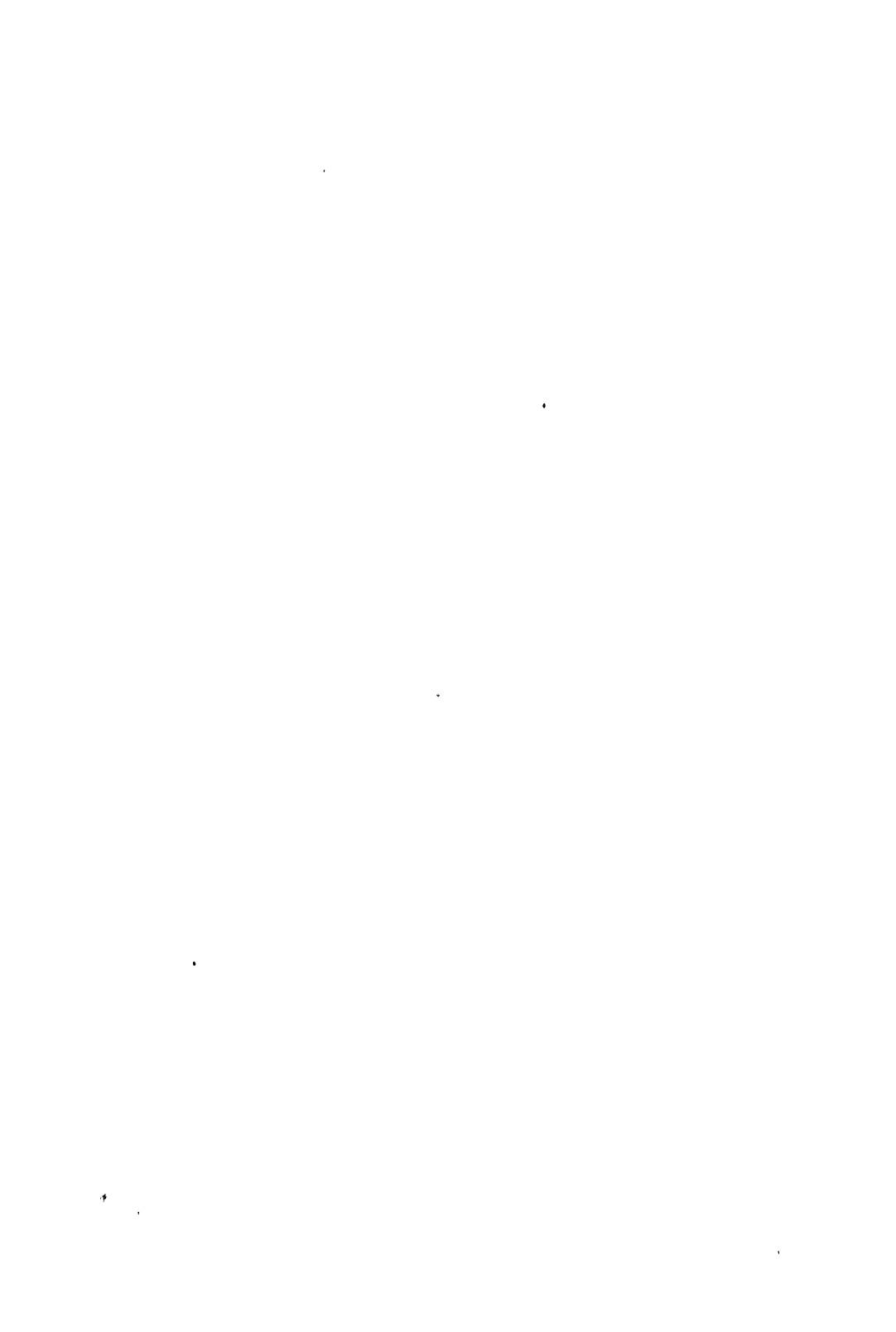
LETTERS TO THE EDITORS

NICOTINAMIDE-CONTAINING NUTRILITES FOR HEMOPHILUS PARAINFLUENZAE*

Sirs:

Except for *Hemophilus influenzae* and *parainfluenzae,* nicotinamide satisfies the essential nutritional requirements for the biosynthesis of the hydrogen-transporting coenzymes for all organisms which do not accomplish this synthesis from simple carbon and nitrogen sources. Codehydrogenases I and II satisfy the factor V requirement of these organisms.[1] In order to determine whether the whole cozymase (codehydrogenase I) molecule or only a portion of it is essential for *Hemophilus parainfluenzae,*[2] we have tested the structural units (nicotinamide, *d*-ribose, adenylic acid) of the molecule individually and together, nicotinamide nucleoside,[3] a pure preparation of cozymase,[4] dihydrocozymase,[5] acid-treated dihydrocozymase[6] (which is completely inactive in *in vitro* enzymatic reactions), and desaminocozymase[7] (a derivative in which the adenylic acid portion of the molecule is replaced by inosinic acid). The latter two compounds do not occur naturally.

The conditions of testing these compounds were similar to those given by Kohn.[8] The results are recorded in the accompanying table.

The oxidized form of the coenzyme is slightly more efficient in promoting growth than the reduced form. This may be due to a difference in permeability of the bacterial cell membrane for the monobasic (oxidized) and the dibasic (reduced) forms of cozymase. Acid-treated dihydrocozymase exhibited some growth-promoting activity, although only in relatively high concentration. Apparently the bacteria can effect some measure of

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[2] We are indebted to Dr. H. I. Kohn for Culture 4101 of the National Collection of Type Cultures, Lister Institute.

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Compounds tested for growth-promoting activity with *Hemophilus parainfluenzae*	Minimum concentration for detectable growth
	γ *mole per ml. medium*
Cozymase	0.2×10^{-5}
Dihydrocozymase....................................	0.3×10^{-5}
Acid-treated dihydrocozymase	0.5×10^{-4}
Desaminocozymase.....	0.5×10^{-5}
Nicotinamide nucleoside..........................	0.1×10^{-4}
" d-ribose, adenylic acid	No growth with 1.0 γ mole per ml. of each substance

resynthesis of cozymase from it. The activity of desaminocozymase (40 per cent as compared with cozymase) corresponds to the similar relative efficiency which we found in the fermentation test. Whether the bacteria utilize the compound as such or resynthesize cozymase from it remains open to question.

The positive result with nicotinamide nucleoside (consisting of nicotinamide and pentose) is most interesting with respect to the biosynthesis of cozymase. Evidently *Hemophilus parainfluenzae* can proceed with the synthesis of cozymase if the first step, the linkage between nicotinamide and pentose, is accomplished. Furthermore, the fact that both the nicotinamide nucleoside and desaminocozymase support growth indicates that the adenylic acid portion of the molecule is of minor importance.

It is remarkable that the highest degree of specialization in biosynthesis of codehydrogenases I and II observed thus far requires but one further chemical linkage than that of most mammals as well as many microorganisms. Finally, it should be mentioned that the significance of these results will have to be taken into account in evaluation of the results obtained by using these organisms for the bioassay of pyridine nucleotide compounds in blood and tissues.

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THE BIOLOGICAL FORMATION OF CHOLESTEROL FROM · ACETIC ACID

Sirs:

The specific precursors from which cholesterol is synthesized by the animal organism are unknown. Earlier results reported from this laboratory[1] suggested a synthesis from small molecules, possibly the intermediates of fat or carbohydrate metabolism. Direct utilization of higher fatty acids to form the sterol molecule was considered quite improbable.

Sonderhoff and Thomas[2] demonstrated that the unsaponifiable fraction of yeast grown on a medium containing deutero acetate had a deuterium content so high that a direct conversion of acetic acid to sterols had to be postulated. The yeast sterols were not identified.

We have, in two experiments, fed deuterium-containing sodium acetate to adult mice and growing rats for 8 days and determined the deuterium content of cholesterol and fatty acids isolated from the animal carcass. Some deuterium oxide was present in the body water as a result of the oxidation of the dietary deutero acetate. The deuterium concentration in the cholesterol samples from both experiments was over 3 times as high as that of the body fluids at the end of the experiment. From experiments in which mice were given heavy water to drink[1] it can be estimated that in a period of 8 days about 20 per cent of the cholesterol will be replaced by newly synthesized material, and that the total cholesterol will then have a deuterium concentration of about 10 per cent of that in the body fluids. In the above experiments the cholesterol has a deuterium concentration at least 30 times higher than would be expected if it had originated in the body water. Acetic acid may therefore act as a precursor in the biological formation of cholesterol.

In the experiment with mice 1.3 per cent of all the hydrogen atoms of the body cholesterol was derived from the hydrogen of the sodium acetate; in the experiment with rats the corresponding value was 0.4 per cent. The cholesterol analyzed represented a pooled sample of that already present and that newly formed. As, in the experiment with mice, only one-fifth of the total cholesterol was synthesized during the 8 day period, 7 per cent of the hydrogen atoms of the newly formed cholesterol must have been derived from the deutero acetate. This represents a minimum value, since

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[2] Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).

the dietary deutero acetate may have been appreciably diluted by normal acetic acid arising from the intermediary metabolism.

The location of the deuterium in the cholesterol molecule is under investigation.

The deuterium content of the fatty acid fraction is about one-half of that of the body fluids. This result furnishes additional support for the view that the higher fatty acids are not intermediates in sterol synthesis, but does not necessarily exclude the utilization of the carbon chain of acetic acid for fatty acid synthesis.

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THE ENZYMATIC ACTION OF MYOKINASE

Sirs:

Myokinase is an acid-stable protein[1] occurring in skeletal muscle; when acting with yeast hexokinase, it brings about the transphosphorylation: adenosine diphosphate + hexose → adenylic acid + hexose monophosphate.[1] It has been found recently[2] that myokinase is also a necessary component in the dephosphorylation of adenosine diphosphate in muscle. Myosin, purified by repeated precipitations, dephosphorylates adenosine *tri*phosphate but not adenosine *di*phosphate.[3] Addition of a few micrograms of myokinase to the system brings about dephosphorylation of adenosine diphosphate.

The mechanism of the myokinase action has so far not been understood. Incubation of adenosine diphosphate with myokinase does not give rise to any change in the labile phosphate. It was found, however, that incubation of adenosine diphosphate with myokinase and adenylic acid deaminase[4] yields ammonia, whereas incubation of the nucleotide with deaminase or myokinase separately does not yield any ammonia. Myokinase apparently forms adenylic acid or some other nucleotide[5] which can be deaminated by Schmidt's deaminase.

Dr. M. Johnson, University of Wisconsin, suggested in a personal communication about 6 months ago that myokinase might catalyze a reversible transfer of phosphate from 1 mole of adenosine diphosphate to another, yielding adenosine triphosphate and adenylic acid. This suggestion has now been proved to be very nearly true. Incubation of adenosine diphosphate with myokinase gives rise to formation of small amounts of adenylic acid and adenosine triphosphate. The adenylic acid was crystallized and identified. The presence of adenosine triphosphate was demonstrated by means of hexokinase or reprecipitated myosin. Incubation of adenosine diphosphate with myokinase and deaminase yields adenosine triphosphate and inosinic acid. The table shows that adenosine triphosphate and adenylic acid (or inosinic acid) are formed in approximately equimolar amounts. The conversion of adenosine diphosphate to the two

[1] Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **137**, 789 (1941).
[2] Kalckar, *Proc. Am. Soc. Biol. Chem.*, in press (1942).
[3] Ljubimova and Pevsner, *Biokhimiya*, **6**, 178 (1941).
[4] Schmidt, *Z. physiol. Chem.*, **179**, 243 (1928).
[5] Kiessling and Meyerhof, *Biochem Z.*, **296**, 410 (1938).

nucleotides does not go very far (about 25 per cent is converted in the presence of myokinase, and about 50 per cent in the presence of both myokinase and deaminase).

Conversion of Adenosine Diphosphate to Adenosine Triphosphate and Adenylic Acid

Adenosine diphosphate (ADP) was incubated with myokinase or deaminase or both. Ammonia was determined on an aliquot of the trichloroacetic acid filtrate, and to the remainder were added barium acetate and sodium hydroxide to alkaline reaction. The barium precipitate which contains ADP and adenosine triphosphate (ATP) was analyzed for the latter by the hexokinase test, and for pentose. The barium supernatant which contains adenylic (or inosinic) acid was analyzed for organic phosphorus.

		Barium precipitate		Barium supernatant	
ADP corresponding to 300 γ labile P; volume of reaction mixture, 0.7 ml. (incubation, 30 min. at 30°). Myokinase, 10 γ protein per ml.; deaminase, 500 γ protein per ml.	Ammonia	P difference calculated from pentose	ATP, disappearance of labile P in hexokinase test	P difference found	P difference calculated from ammonia
	γ N	γ	γ	γ	γ
ADP + deaminase..	0		15*		
" + myokinase..	0	−41	53.5	+51	
" + " + deaminase.	36	−80	53.0	+83	+80

* Probably there were minute traces of myokinase in the deaminase.

When adenosine triphosphate and adenylic acid are incubated with myokinase, about 20 per cent of the two nucleotides is converted into adenosine diphosphate and then the reaction stops. The phosphate dismutation, 2 adenosine diphosphate $\rightleftharpoons$ 1 adenosine triphosphate + 1 adenylic acid, is apparently not a simple equilibrium. Some other compound must be formed as an intermediate product.

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CREATINE FEEDING AND CREATINE-CREATININE EXCRETION IN MALES AND FEMALES OF DIFFERENT AGE GROUPS

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In the experiment of Rose, Ellis, and Helming (1) in which approximately 1.0 gm. of creatine was administered daily for 7 weeks to a young male and a young female adult, certain differences in the fate of the exogenous creatine were observed. It seemed of interest to determine whether dissimilarities in creatine metabolism attributable to age, sex, or musculature would be observed if creatine were administered over a period of time to male and female representatives of different age groups.

EXPERIMENTAL

The following subjects were used: Subject 2, a young man accustomed to rather vigorous exercise; Subjects 3 and 4, two young men of average musculature; Subject 5, a young man with muscular dystrophy and atrophy of several muscles; Subject 6, a healthy man of 70; Subject 8, a young woman accustomed to rather vigorous exercise; Subjects 9, 10, and 11, three young women of average musculature; Subject 12, a woman of 39, in good health, who 2 years earlier had had a bilateral oophorectomy without implants; Subject 13, a healthy woman of 59 who had been physically active in her youth, and who had passed the climacteric 8 years earlier; Subject 14, a second healthy woman of 59 who had passed the climacteric 4 years earlier; Subject 10', the woman who 16 years earlier had been Subject 10, and who had passed the climacteric 2 years before this experiment; Subjects 15 and 16, 10 year-old boy and girl twins. Data from the study of Rose and coworkers (1) are included as Subject 1, a young man accustomed to vigorous exercise, and Subject 7, a young woman who exercised vigorously each summer.

For a few days preceding and throughout the experiment, the subjects ate well balanced, meat-free diets of constant composition (with the exceptions mentioned below). After the 5th day of the fourth ingestion period of Subject 2, 40 gm. of sugar were added daily to his diet, since he had felt hungry although his diet supplied 3310 calories and 16.4 gm. of nitrogen. In order to determine the effect of high protein ingestion upon the retention of exogenous creatine, the diet of Subject 3 was changed for the eleventh ingestion period only to one somewhat more than 3-fold
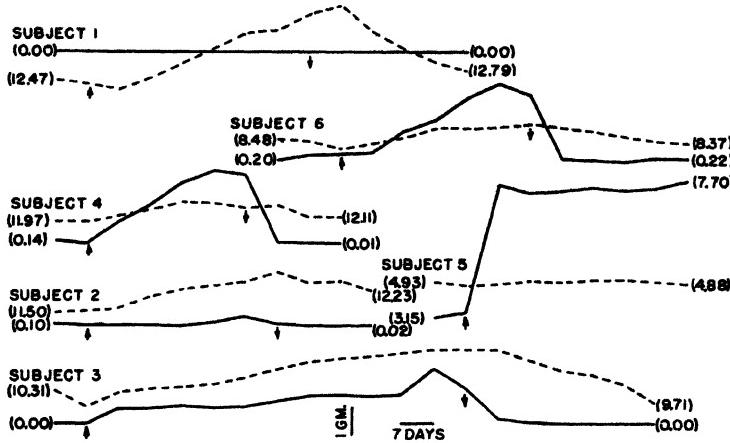
FIG. 1. Curves of 7 day urinary creatine-creatinine of adult males on a meat-free diet supplemented with creatine. The solid lines denote creatine output; the broken lines creatinine. The numbers in parentheses denote the gm. of creatine or creatinine excreted during the initial and final 7 day periods. The upward pointing arrows indicate the time of addition of creatine to the diet; the downward pointing arrows the time of its withdrawal. Subject 1 (1). Subject 2 ingested 7.6 gm. of creatine during his fifth ingestion period instead of the usual 7.0 gm. Subject 3 ingested 0.5 gm. of creatine daily for 5 days preceding his daily ingestion of 1.0 gm. During his eleventh period of creatine ingestion his diet contained over 3 times as much protein as during the other periods.
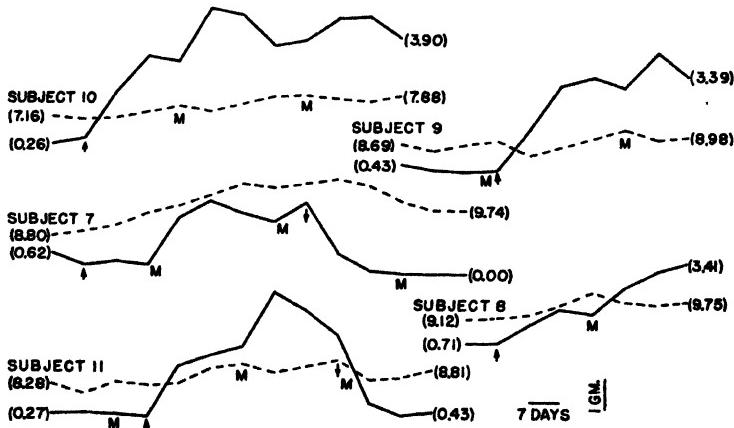
FIG. 2. Curves of 7 day urinary creatine-creatinine of normal, young women on a meat-free diet supplemented with creatine. The solid lines denote creatine output; the broken lines creatinine. The numbers in parentheses denote the gm. of creatine or creatinine excreted during the initial and final 7 day periods. The upward pointing arrows indicate the time of addition of creatine to the diet; the downward pointing arrows the time of its withdrawal. The letter M indicates menstrual periods. Subject 7 (1). Subject 10 ingested 0.5 gm. of creatine daily for 10 days preceding her daily ingestion of 1.0 gm. Subject 11 ingested 7.2 and 7.4 gm. of creatine during her fourth and fifth creatine ingestion periods respectively instead of the usual 7.0 gm.

higher in protein. The diets of Subjects 5 and 10 were not invariably constant, though practically so, and were at all times free of meat and of meat extractives.
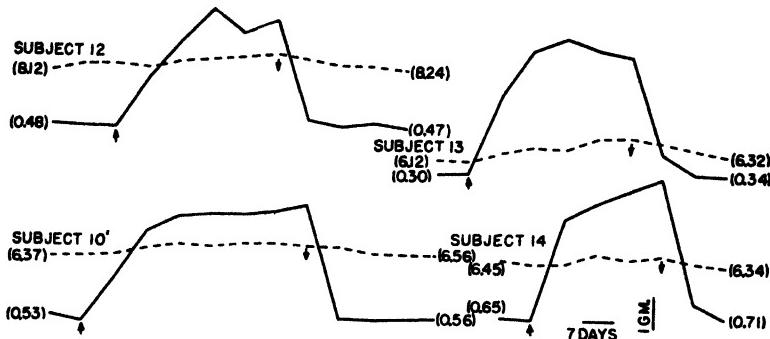
FIG. 3. Curves of 7 day urinary creatine-creatinine of non-menstruant, adult females on a meat-free diet supplemented with creatine. The solid lines denote creatine output; the broken lines creatinine. The numbers in parentheses denote the gm. of creatine or creatinine excreted during the initial and final 7 day periods. The upward pointing arrows indicate the time of addition of creatine to the diet; the downward pointing arrows the time of its withdrawal. Subject 10′ is Subject 10 (Fig. 2) after a period of 16 years. Subject 12 had a bilateral oophorectomy without implants 2 years preceding this experiment. The other subjects had passed the normal climacteric.
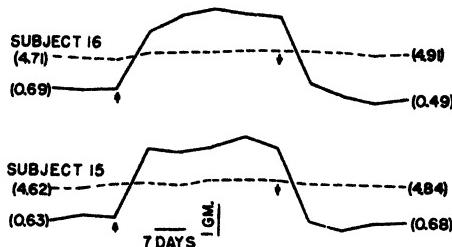
FIG. 4. Curves of 7 day urinary creatine-creatinine of 10 year-old twins on a meat-free diet supplemented with creatine. The solid lines denote creatine output; the broken lines creatinine. The numbers in parentheses denote gm. of creatine or creatinine excreted during the initial and final 7 day periods. The upward pointing arrows indicate the time of addition of creatine to the diet; the downward pointing arrows the time of its withdrawal. Subject 15 is male, Subject 16 female.

In each case there was a preliminary period which served to show the normal excretion of creatine and creatinine by each subject; a creatine ingestion period; and, in all but three cases (Subjects 5, 8, and 9), an after

period.   Creatine, C.P., obtained from the Eastman Kodak Company and recrystallized was used.   The creatine was assayed by determining the amount of creatinine it formed upon dehydration and, in some instances, by determining its nitrogen content.   Analyses showed it to be free of creatinine.

During the ingestion periods 1.0 gm. of anhydrous creatine (Subject 3, 9, and 10) or the equivalent in creatine hydrate was administered daily to

TABLE I

Calculated Creatine Retained and "Retained Creatine" Changed to Creatinine by Adult Males during First 5 Weeks of Creatine Ingestion

The figures for creatine are expressed as creatinine.   The creatinine coefficient given is that of the period preceding the ingestion of creatine.   Extra creatinine refers to the difference between the preformed creatinine output expected, based upon the output of the preliminary periods, and the total preformed creatinine output during the experimental period of 7 weeks.   The term "retained creatine" denotes the creatine not excreted as such.

Subject No.	Muscle status	Creatinine coefficient	Creatine ingested per kilo body weight	Creatine retained per kilo body weight	Ingested creatine retained	Extra creatinine excreted	"Retained creatine" excreted as creatinine
			gm.	gm.	per cent	gm.	per cent
1.* Young male, 72.5 kilos, height 177.8 cm.	Above average	24.5	0.40	0.40	100.0	3.17	10.9
2. Young male, 58.4 kilos, height 170.2 cm.	" "	28.2	0.53	0.52	98.9	3.30	10.87
4. Young male, 67.4 kilos, height 182.9 cm.	Average	25.4	0.45	0.32	72.2	2.19	10.0
5.† Young male, 59.0 kilos, height 182.2 cm.	Poor	11.8	0.51	0.16	30.5	0.00	0.0
6. 70 yr. male, 62.2 kilos, height 167.6 cm.	Average	18.9	0.49	0.38	79.1	2.14	9.0

* Taken from the experiments of Rose and coworkers (1).
† With muscular dystrophy.

the adults with the exceptions described below; creatine hydrate equivalent to 0.5 gm. of creatine was administered daily to the children.   The creatine was taken in milk or in water in two equal doses, one with breakfast, one with luncheon, except in the experiments with Subjects 3, 9, and 10.   In these cases the entire dose was taken with luncheon.   In the experiments with Subjects 3 and 10 the daily ingestion of 0.5 gm. of creatine preceded for 5 and 10 days respectively the daily ingestion of 1.0 gm.   During the

fifth ingestion period Subject 2 ingested 1.10 gm. of creatine daily for 5
days and 1.04 gm. daily for 2 days.   During the last 2 days of the fourth

TABLE II

*Calculated Creatine Retained and "Retained Creatine" Transformed into Creatinine by
Adult Females during First 5 Weeks of Creatine Ingestion*

The figures for creatine are expressed as creatinine.   The creatinine coefficient
given is that of the period preceding the ingestion of creatine.   Extra creatinine
refers to the difference between the preformed creatinine output expected, based
upon the output of the preliminary periods, and the total preformed creatinine output
during the experimental period of 7 weeks.   The term "retained creatine" denotes
the creatine not excreted as such.

Subject No.	Muscle status	Creatinine coefficient	Creatine ingested per kilo body weight	Creatine retained per kilo body weight	Ingested creatine retained	Extra creatinine excreted	"Retained creatine" excreted as creatinine
			gm.	*gm.*	*per cent*	*gm.*	*per cent*
7.* Young female, 72.5 kilos, height 172.7 cm.	Above average	17.8	0.40	0.34	84.2	4.93	20.1
8. Young female, 64.5 kilos, height 157.5 cm.	" "	20.3	0.47	0.36	76.6	2.48	10.7
9.† Young female, 70.7 kilos, height 172.7 cm.	Average	17.7	0.43	0.23	54.2	0.65 / 0.23	4.0 / 1.4
10. Young female, 60.3 kilos, height 157.5 cm.	"	16.9	0.57	0.30	52.5	0.86	4.7
10.' No. 10 after 16 yrs., 2 yrs. after menopause, 56.4 kilos	"	16.2	0.54	0.29	54.0	1.57	9.6
11.† Young female, 54.6 kilos, height 167.6 cm.	"	21.9	0.56	0.34	59.7	2.52 / 2.07	13.8 / 12.0
12.‡ 39 yr. female, 75.0 kilos, height 162.6 cm.	"	15.9	0.40	0.21	53.2	0.85	5.3
13. 59 yr. female, 70.7 kilos, height 152.4 cm., 8 yrs. after menopause	"	12.4	0.43	0.17	40.2	2.48	20.4

* Taken from the experiment of Rose and coworkers (1).
† Calculations based on a fore period of 28 and of 14 days in the order named.
‡ 2 years after bilateral oophorectomy without implants.

and the first 2 days of the fifth ingestion period, Subject 11 ingested 1.10 gm.
of creatine daily; during the following 4 days she ingested 1.04 gm. daily.

In the experiments with Subjects 2, 8, 11, 12, 13, and 10′ the urinary inorganic phosphorus was determined daily. The methods of urine collection and the analytical procedures used in determining the daily output of urinary nitrogen, total and preformed creatinine, and phosphorus were those described earlier (2). Qualitative tests made for pathological constituents in the urine were always negative.

The successive 7 day urinary output of creatine and creatinine by each of the subjects is shown in Figs. 1 to 4. Time in 7 day periods is plotted as abscissae, and gm. of urinary creatine and creatinine excreted during the corresponding 7 days as ordinates. In Tables I to III are summarized data regarding the amounts of creatine ingested and metabolized during

TABLE III

Calculated Creatine Retained and "Retained Creatine" Transformed into Creatinine by 10 Year-Old Twins during First 5 Weeks of Creatine Ingestion

The figures for creatine are expressed as creatinine. The creatinine coefficient given is that of the period preceding the ingestion of creatine. Extra creatinine refers to the difference between the preformed creatinine output expected, assuming that the initial creatinine coefficient would be maintained in relation to the proportion of increased weight which might be muscle tissue, and the total preformed creatinine output during the experimental period of 7 weeks.

Subject No.	Muscle status	Creatinine coefficient	Creatine ingested per kilo body weight	Creatine retained per kilo body weight	Ingested creatine retained	Extra creatinine excreted	"Retained creatine" excreted as creatinine
			gm.	gm.	per cent	gm.	per cent
15. 10 yr. boy, 31.5 kilos, height 137.8 cm.	Average	21.6	0.48	0.13	27.9	0.39	9.3
16. 10 yr. girl, 31.4 kilos, height 139.7 cm.	"	21.1	0.48	0.12	25.7	0.74	19.1

the first 5 weeks by each of those subjects who ingested creatine for 5 weeks (this precludes Subject 14), and who had a fore period of 2 (Subject 10, approximately 2) weeks (this precludes Subject 3). Quantities of creatine are always expressed in terms of creatinine values. The difference between the preformed creatinine output expected, based upon the fore period output, and the total preformed creatinine excreted during the 7 weeks is called extra creatinine. For Subjects 9 and 11 calculations based on both a fore period of 4 weeks and, as with all of the other subjects, a fore period of 2 weeks are given to illustrate that the creatine metabolism of these young women varied throughout a menstrual cycle to such an extent that there is a considerable difference in the calculated amounts of extra creatinine depending upon the extent of the fore period used. Data for

longer fore periods were not available in the other cases. The term "retained creatine" denotes the creatine not excreted as such.

DISCUSSION

Experiments with Adults

In Regard to Retention of Ingested Creatine—Since approximately the same amount of creatine (1.0 gm.) was ingested daily by each subject, the percentage of creatine retained by each during the first 5 weeks of ingestion will serve to show his creatine tolerance. With the exception of Subject 1 (1), the tolerance of each was exceeded. Subject 1 ingested somewhat less creatine in proportion to body weight than did the other males. The young men who were accustomed to vigorous exercise surpassed the other subjects in creatine tolerance and in the amount of creatine retained in proportion to body weight. Next in order of creatine tolerance was one of the young females with well conditioned muscles. However, in respect to the amount of creatine retained per kilo of body weight, she was surpassed by the other young woman with above average musculature, and by the 70 year-old man, and equaled by a thin young woman with average musculature, although their creatine tolerances were from 5 to 25 per cent less. The muscular dystrophic male and the 59 year-old women showed the lowest tolerance for the exogenous creatine. In this respect Subject 13 who had been somewhat athletic in her youth was not different from her less active contemporary during the 4 weeks in which the latter ingested creatine. The tolerance of the oophorectomized woman was equivalent to the tolerances of the normal young women with average musculature. 16 years of aging and the cessation of the menses did not diminish the ability of the middle-aged woman to retain the ingested creatine.

These data do not suggest any correlation between normal creatinuria on meat-free diets and capacity to retain exogenous creatine. The 70 year-old man retained more creatine than one of the normal young men and more than the women, with one exception, and yet his normal creatinuria exceeded that of the young man and was comparable to that of the young women. The normal creatinurias of the oldest women were in the range of those of the younger women who showed a much greater creatine tolerance. It is puzzling that persons who exhibit creatinuria on diets practically free of creatine should retain relatively large amounts of ingested creatine. An explanation may be that some of the difference between the total and preformed creatinine, reported as creatine, is due to other substances which respond to the Jaffe reaction. In some experiments carried out by the author significantly greater amounts of so called creatine were excreted by two subjects when extra ascorbic acid, either as such or as orange juice, was added to their otherwise constant diet.

The oldest women and the young male with muscular dystrophy had the lowest creatinine coefficients.[1] However, no other example of a parallelism between creatine tolerance and creatinine coefficient can be observed from these data.

In Regard to Transformation of "Retained Creatine" into Creatinine—The increased excretion of preformed creatinine during the ingestion of creatine, the continuance of this increase for several days of the after period, and then its gradual decrease to the fore period level indicate an increased dehydration of creatine associated with its ingestion. Since some of the subjects changed somewhat in body weight, an error is involved in calculating the expected preformed creatinine output on the basis of the amount excreted during the preliminary periods. However, the only appreciable weight changes were those of Subject 11, who gained 3.1 kilos if the 4 week fore period is considered, and of Subject 10', who gained 2.3 kilos.

The amounts of extra creatinine excreted as well as the amounts of "retained creatine" changed to creatinine differed considerably. In general those who retained the largest amounts of the creatine supplement excreted the largest amounts of extra creatinine; yet these data do not indicate any consistent relationship between creatine retention and dehydration. The muscular dystrophic male exhibited no continuous increase in preformed creatinine excretion, while the amount of extra creatinine excreted by one 59 year-old woman (Subject 13) was in the range of amounts excreted by one young man, by the 70 year-old man, and by two young women each of whom showed a much greater creatine retention. The amount of extra creatinine excreted by the oophorectomized woman was small but that was also true in the case of two normal young women.

On the basis of these data the creatinine coefficient of an individual is not an index of ability to dehydrate exogenous creatine.

In Regard to Fluctuations in Urinary Creatine and Creatinine—In the case of the young women the premenstrual periods which occurred after several days of creatine ingestion were characterized by decreases in creatinuria, and the postmenstrual periods by striking increases in creatinuria. This suggests a relationship between the menstrual cycle and creatine metabolism. However, there were some fluctuations in the creatinuria of the oophorectomized woman, of the non-menstruant women, of some of the men, and of the children.

The output of preformed creatinine by the young women was not as regular as that of the men. In some cases there was a definite decrease during a postmenstrual period, but these decrements were not equivalent to the extra creatine excreted at such times.

[1] Mg. of preformed creatinine per kilo per day.

In Regard to Urinary Inorganic Phosphorus—No uniformity in the variation in urinary inorganic phosphorus associated with the retention of supplementary creatine was observed. The urinary inorganic phosphorus of Subjects 11 and 13 increased somewhat during the experiment. The total urinary inorganic phosphorus of Subjects 2, 8, 10', and 12 was 2.28, 5.62, 1.72, and 2.50 gm. respectively less than the amount expected according to the fore period output of each, and the creatine retained (expressed as creatine not excreted as such or as extra creatinine) by these subjects was 31.37, 23.95, 17.09, and 17.63 gm. in the order named. The urinary inorganic phosphorus of Subject 2 returned to the fore period level during the after period; that of Subjects 10' and 12 did not, although in each case it increased somewhat. There was no after period in the experiment with Subject 8.

Experiments with 10 Year-Old Twins

In Regard to Retention of Ingested Creatine—It is interesting that the boy and girl did not differ significantly in ability to retain exogenous creatine. Each gained in weight and in height during the experiment. If new muscle cells constituted 40 per cent of the gain in body weight, sufficient creatine was retained by the boy to supply this muscle tissue, but in the case of the girl at least 50 per cent of the creatine for such an amount of newly acquired muscle tissue would have had to be supplied from some other source than the exogenous creatine.

In Regard to Preformed Creatinine Output—The creatinine coefficients of these twins were at an adult level. In each case during the ingestion of creatine the preformed creatinine output increased slightly; during the after period it decreased but did not drop back to the fore period value. One would expect the creatinine coefficients of children to be maintained and possibly to increase as growth occurs. The extra creatinine of Table III is the actual extra preformed creatinine, as determined in the case of the adults, corrected on the assumption that in each case 0.4 of the increase in body weight was muscle tissue, and that the initial output of preformed creatinine in proportion to body weight was maintained throughout the experimental period.

SUMMARY

The daily urinary creatine, creatinine, and, in six cases, inorganic phosphorus were determined before, during, and after the long continued administration of approximately 1.0 gm. of creatine daily to a representative group of men and women. Data from the experiment of Rose, Ellis, and Helming (1) are included.

Two young males who were accustomed to rather vigorous physical

exercise retained more exogenous creatine than any of the other subjects. One of the women who was accustomed to vigorous exercise each summer exceeded the other women and two young men of average musculature in creatine tolerance. The young man with muscular dystrophy and the 59 year-old women showed the lowest creatine tolerance.

No correlation was shown between, on the one hand, the capacity to retain exogenous creatine and, on the other hand, the extent to which the retained creatine was dehydrated, the degree of creatinuria on a creatine-free diet, or the creatinine coefficient.

The ability to transform ingested creatine into creatinine varied irrespective of age, sex, muscle development, creatinine coefficient, or degree of creatinuria on a creatine-free diet. The creatinuria of adults, old or young, may not be explained by an inability to dehydrate exogenous creatine.

Since most of the subjects exhibited some irregularity in the total 7 day output of creatine, the striking fluctuations exhibited by the young women in premenstrual and postmenstrual periods cannot be attributed exclusively to the menstrual cycle.

Neither 16 years of aging nor the cessation of the menses had any significant effect upon the creatinine coefficient or the creatine tolerance of one woman.

Decreases in urinary inorganic phosphorus, which were observed associated with the retention of creatine in some of the experiments in which the phosphorus output was studied, bore no apparent relation to the amount of supplementary creatine retained.

0.5 gm. of creatine was administered daily for 5 weeks to a pair of boy and girl twins, age 10 years. Each retained about one-fourth of the ingested creatine, and in each case an increased output of preformed creatinine was associated with the ingestion of creatine.

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SEXUAL HORMONES IN ACHLYA

IV. PROPERTIES OF HORMONE A OF ACHLYA BISEXUALIS

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The sexual reproductive process of *Achlya ambisexualis*, a heterothallic form belonging to a well known and widely distributed genus of saprophytic aquatic fungi, is initiated and coordinated by means of specific substances (Raper, 1939, *a*). Four of these substances have been demonstrated (Raper, 1939, *b*, 1940) in the rôles of initiators and coordinators of the various stages in the sexual process.

The entire process is initiated by the secretion from the female plant of hormone A which brings about the formation of male sexual organ initials, thin thread-like filaments or antheridial hyphae, on the male plant. The antheridial hyphae then secrete the second of the specific substances, hormone B, which causes the formation of female sexual organ initials, oogonial initials, on the female plant. The oogonial initials, when fully formed, secrete the third substance, hormone C, which has two specific actions on the antheridial hyphae of the male: (1) It causes a directional growth of the antheridial hyphae, so that they grow to and become applied to the oogonial initial, the locus of the production of hormone C. (2) Once the tip of an antheridial hypha becomes applied to the walls of an oogonial initial, hormone C is necessary for the differentiation of the small male sexual organ, the antheridium. Finally, after they are differentiated from the antheridial hyphae, the antheridia secrete a fourth specific substance, hormone D, which brings about the differentiation of the female sexual organ, the oogonium, from the oogonial initial. The differentiation of gametes and the process of fertilization follow to form oospores (zygotes).

The discovery of this mechanism pointed to a number of possible lines of investigation. Of these possibilities, that relating to the chemical nature and identity of the several specific substances was particularly interesting to the authors of the present account. The first of the four

specific substances, hormone A, has been the subject of this work for three reasons. (1) Of the four hormones it alone is secreted by the entire vegetative plant and not by specialized organs; hence probably in the greatest quantity. (2) Its activity in controlling the initial reaction in the sexual process makes it of first importance. (3) A method of quantitative determination, biological assay, for this substance has already been worked out (Raper, 1942).

The present paper reports the results of the work done on the problem of the properties of hormone A during the stay of the senior author in these laboratories. The work is being continued at present i⁻. these laboratories.

Materials and Methods

It is known from previous work that two species of *Achlya* in which hormones have been demonstrated, *A. ambisexualis* and *A. bisexualis*, are incompatible. Incompatibility results from specificity of certain of the hormones secreted by the two plants. Hormone A of either species brings about the production of antheridial hyphae on the male plants of both species. However, the male plant of *A. ambisexualis* is the more vigorous of the two and it reacts much more strongly in the filtrate from the female plant of *A. bisexualis* than in that from the female of the species to which it belongs. Whether the greater activity brought about by the filtrate of *A. bisexualis* female is due to minor differences in the chemical nature of the specific substances secreted by the two species or to the greater quantity produced by this plant is an interesting question, for which the answer is not known. In either case the quantitative test for hormone A of *A. bisexualis* is more sensitive than for the corresponding hormone of *A. ambisexualis*, since the method of assaying depends on the intensity of the reaction induced in the male plant.

The female plant of *Achlya bisexualis*, therefore, has been used exclusively as the source of hormone A in the present study. It is the opinion of the writers that the initial hormones of the two plants will eventually be shown to differ slightly from each other in chemical composition.

The male plant of *Achlya ambisexualis* has been used in all cases in testing for hormone A.

Technique of Culturing Female Plant—A large number of natural products of plant and animal origin have been tested with the male test plants in the hopes of finding a source of the hormone other than the female mycelium. In all cases the results have been negative. Preliminary work also showed that the quantity of hormone secreted by the female plant was exceedingly small. As a consequence large amounts of the fungus have of necessity been grown in the laboratory to furnish the material necessary for extended work.

Bottles of 20 liters capacity, such as are used to distribute distilled or mineral water, have been found satisfactory as culture vessels. 18 liters of distilled water containing low concentrations of several salts (KH_2PO_4, $M/3 \times 10^4$; $MgSO_4$, $M/8 \times 10^4$; $ZnSO_4$, $M/10^7$; $CaCl_2$, $M/10^5$; and $FeCl_3$, $M/10^6$) were poured into each bottle. The bottles and the dilute salt solution were then sterilized in a large horizontal drum with live steam at atmospheric pressure for 5 hours. Upon cooling, 100 gm. of halved hempseed[1] and 250 cc. of water in which they had been boiled were added to each bottle. At the same time a sterilized solution of 350 to 400 mg. of malonic acid was added to each bottle, the addition of this acid in the concentration of 20 to 30 mg. to each liter of culture medium having been shown to increase greatly the production of hormone A by the female plant (Raper, 1942). The culture was then inoculated by adding to each bottle a fully matured mycelium of the female plant grown on a half hempseed in a small Erlenmeyer flask in 10 cc. of water. Such a mycelium has a large number of sporangia and chlamydospores which within a few hours liberate millions of zoospores, each capable of germinating to form a mycelium.

A sterile aeration tube was placed in each bottle with sterile cotton forming a plug. A piece of pressure tubing was used to connect the aeration tube to an outlet of the compressed air line. The upper portion of the aeration tube was packed with glass wool to entrap any microorganisms blown into it from the air line. For the first 2 or 3 days of incubation only a small stream of air was blown through the culture and the hempseed remained at the bottom of the bottle and became uniformly covered with the young germlings. A strong stream of air was then blown into the culture until the somewhat matted mass of seed and fungus was broken up into individual seeds and their attached mycelia. The cultures were then incubated for 10 to 12 additional days with a sufficient stream of air bubbled through them to keep the mycelia separated and in constant motion. The air stream method of stirring also served the necessary function of keeping the water well aerated, a constant supply of oxygen being required for the vigorous growth of the organism. At the end of the incubation period the mycelia and seeds were removed from the liquid by filtering through a pad of cotton, the filtrate containing 500 to 1500 units of hormone A per cc.

From the standpoint of hormone A content of these large cultures sterilization of the medium and the culture of the plant under aseptic conditions

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[1] The seeds were neatly split in quantity by a machine designed and built by Mr. Tomlin. Seeds boiled until the seed coats burst were found to be very inferior to split seed boiled only long enough to effect sterilization. On halved seeds the mycelia grew readily and vigorously.

are not absolutely necessary. When these precautions are not taken the fungus gets well started before bacterial contamination becomes apparent and the vigor of mycelial growth is only slightly affected. The hormone content of such a culture is decreased by a negligible amount. However, the filtrate from contaminated cultures is so unpleasant to work with that the extra time and effort required to grow the source plant in pure culture are well spent.

Biological Assay—The method of quantitative determination of hormone A has been described in a previous paper (Raper, 1942). The intensity of the initial male reaction, as measured by the relative number of antheridial hyphae produced on a male plant, is directly proportional to the concentration of hormone A. A physiological unit of hormone A has been defined as that amount of hormone per cc. of water which will bring about the production of an average of ten antheridial branches per 3 mm. of hyphal tip on 72 hour male tests plants, at 25°, at pH 6.0, when the concentration of dissolved electrolytes does not exceed 0.001 M, and at the time of maximal male sensitivity. Light has not been shown to affect the reaction.

One modification has been made in the test. It was shown that the sensitivity of the male test plants to hormone A varies greatly at different times. This variation is not a strict diurnal rhythm and no means has been found whereby it can either be eliminated or stabilized at 24 hours. It has been found, however, that plants left in a hormone A solution for 24 hours produce no greater number of antheridial hyphae than are produced in the same solution in a 2 hour period at the time of maximal sensitivity. After 24 hours in the test solution those antheridial hyphae produced first are extensively branched but only the original branches (arising directly from the parent vegetative hyphae) were counted. Even so there was still slight variation from day to day, but this variation seldom exceeded 25 per cent deviation from the mean. To correct for this variation, controls containing hormone A in the concentrations of 0.6, 6.0, and 60 hormone A units per cc. were tested simultaneously with each unknown sample. These control solutions were respectively 10^{-5}, 10^{-4}, 10^{-3} dilutions of the acetone-soluble material from concentrated female filtrate. This standard solution, used throughout the work, contained 6×10^{4} hormone A units per cc. Each sample was tested in a series of at least five dilutions, a factor of 10 between the concentration in the members of the series. From the counts thus obtained the amount of hormone A (in physiological units) contained in any sample could be calculated.

Properties of Hormone A

. A number of attempts have been made during 2 years to isolate hormone A as a chemically pure compound. While this objective has not been at-

tained, many pertinent facts about the properties of the hormone have been determined. Those most likely to be of help in future work on isolation are given below.

Solubilities—The solubilities of the active substance in the more common organic solvents are as follows: (1) very soluble in methyl alcohol, ethyl alcohol, acetone, diethyl ketone, ethylpropyl ketone, dioxane, acetic acid, acetic anhydride, methyl acetate, pyridine, and chloroform; (2) slightly soluble in ethyl acetate, propyl alcohol, amyl alcohol, ethyl ether, carbon tetrachloride, toluene (90–100°), and water (100°); and (3) insoluble in toluene (cold), water (cold),[2] petroleum ether, ligroin, benzene, and carbon disulfide.

Metallic Precipitation—Hormone A is not precipitated by ions of heavy metals. Since the active substance is not soluble in water, a modification of the usual procedure has been tried. To an alcoholic solution containing the active substance was added an equal volume of saturated solution of the acetate of the metal in alcohol. This mixture was then diluted with 3 times its volume of distilled water and cooled to 0–5° for 24 to 48 hours. When this procedure was used, the acetates of cadmium, lead, copper, barium, and mercury, as well as phosphotungstic acid, all gave precipitates. In all cases, however, the greater percentages of activity remained in the filtrates. Only in the case of phosphotungstic acid precipitation (in the presence of sulfuric acid) did recovery approximate 100 per cent. The loss of activity in the other instances was apparently due to adsorption of the active compound on the precipitates which were formed. The only satisfactory eluting solvents, alcohol and acetone, however, dissolved a large portion of the precipitates. Thus of the heavy metals tried only phosphotungstic acid can be used to advantage in the purification of the hormone.

Adsorption—The active substance can be adsorbed on a number of materials. Adsorption on norit is very strong from water (very low concentration of the hormone) and ethyl ether. However, acetone elutes only about 25 per cent of that adsorbed, and with a mixture of acetone-water-ammonium hydroxide a maximum of 50 per cent is recovered. Adsorption is also strong on aluminum oxide from water, ether, chloroform, and carbon tetrachloride. From this material the activity can be recovered quantitatively by elution with acetone. On silica hydrate adsorption is slight from ether and carbon tetrachloride and the recovery is complete. There was no adsorption on calcium carbonate from any of the solvents used. Considerable enrichment of the active principle could be attained by ad-

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[2] Hormone A is physiologically active in exceedingly low concentration and, of course, it is sufficiently soluble in cold water to give a solution of the necessary concentration for maximal physiological activity.

sorption on aluminum oxide. On this material the active and a large portion of the inert material was adsorbed at the same level in a Tswett column and enrichment depended almost entirely on the fact that the active material can be eluted with acetone, whereas a stronger eluting agent is required to remove the inactive material.

When a Tswett column of aluminum oxide on which the material had been adsorbed was examined under ultraviolet light, no fluorescence could be detected.

Acid and Base Relations—Hormone A is stable to both acid and base. When solutions containing the active principle were treated with 10 per cent H_2SO_4 and 10 per cent KOH for 24 hours at 25°, no decrease in activity resulted in either case.

Likewise the hormone is soluble neither in dilute acid, nor in dilute base. The active principle is neutral, since it cannot be extracted from ether solution either by repeated partitions with 5 per cent KOH or with 5 per cent HCl.

Ketone and Aldehyde Reactions—When an alcoholic solution containing the active substance is treated with trimethylacethydrazide ammonium chloride (Girard and Sandulesco, 1936) according to the procedure of Petit and Tallard (1939), only 30 to 40 per cent of the activity could be extracted from the alcoholic reaction mixture (non-ketone fraction). However, after hydrolysis for 1 hour with 0.5 N HCl, the remaining 60 to 70 per cent of the active material was extractable in ethyl ether (ketone fraction). When the non-ketonic fraction was treated a second time with the reagent, practically all of the activity was again found in the non-ketonic fraction, the activity in the ketonic fraction being negligible.

These results indicate two possibilities: (1) Two compounds, one ketonic, the other non-ketonic, each having activity in inducing antheridial hyphal formation are secreted by the female plant. (2) A single active compound, a ketone, is present but its separation was incomplete.

The second of the two possibilities seems likely in view of the fact that no other indication of the presence of two active compounds has been encountered elsewhere in the work.

Another indication of the ketonic nature of hormone A is that complete inactivation can be brought about by reaction with 2,4-dinitrophenylhydrazine. A gummy precipitate is formed with this reagent, but neither the precipitate nor the filtrate (from which the excess reagent had been removed) gave any effect when tested with male plants.

Inactivation—Two means have been found of inactivating hormone A in addition to that mentioned above. Temperatures in excess of 130° bring about complete inactivation. In attempts to purify the material by micro distillation *in vacuo* (0.01 mm. of Hg), fractions distilling over below

this temperature were inactive, and at 125–130° the residues were likewise inactive.

A more thermostable derivative was desired and a reaction with diazomethane was carried out. Complete inactivation resulted and no means was found of recovering the activity.

TABLE I

Procedure of Chemical Fractionation of Female Filtrate for Enrichment of Hormone A

Treatment	Active fraction	Dry weight	Total activity, hormone A units × 10⁶	Hormone A units per mg.	Inactive fraction
		gm.			
	Original filtrate	393	1872	4.7×10^3	
Vacuum-distilled	Crude concentrate	393	1872	4.7×10^3	Distillate
Acetone	Filtrate	163	1840	1.13×10^4	Ppt.
Saturated NaCl	Acetone	53	1834	3.45×10^4	Water
Petroleum ether extraction	Water	48	1834	3.75×10^4	Petroleum ether
Ethyl ether extraction	Ether	4.5	1830	4.0×10^5	Water
5% HCl wash	"	4.1	1830	4.5×10^5	HCl
5% KOH "	"	0.554	1830	3.3×10^6	KOH
Toluene, 100°	Soluble		1774		Residue
Chilled to 0°	Ppt.	0.113	1700	1.54×10^7	Filtrate
Phosphotungstic acid	Filtrate	0.063	1625	2.6×10^7	Ppt.
Adsorbed on Al₂O₃	Adsorbed		1600		Filtrate
Eluted with acetone	Eluate	0.013	1575	1.21×10^8	Residue
Dissolved in chloroform, ether-petroleum ether added at −80°	Ppt.	0.0042	700	1.66×10^8	Filtrate
Dissolved in H₂O at 100°	Water	0.002 (Ca.)	700	3.5×10^8	Residue

Chemical Fractionation

A representative chemical fractionation will be given to illustrate the procedure followed in attempts to isolate the active compound. The procedure is outlined in Table I.

The filtrate from eighty 18 liter cultures of the female plant (1440 liters) was collected over a period of 2 months. The hormone content varied between 1000 and 1500 hormone A units per cc. of filtrate with an average of 1300 hormone A units per cc. The starting material thus contained 1.872×10^9 units of hormone A.

Concentration—Each week's collection of filtrate (180 liters) was concentrated in a large metal still (with continuous feed) at 25 to 30 mm. pres-

sure to approximately 1/200 its original volume.   The solid material in the combined concentrates of the 8 weeks weighed 393 gm. and no activity was lost during concentration.

Acetone Precipitation—To the 7 liters of concentrate was added an equal volume of redistilled acetone and the temperature of the mixture was lowered and kept at 0° for a week.   A precipitate of 230 gm. contained 3.2 $\times 10^7$ units.   The filtrate contained 163 gm. of solid material which had a total activity of 1.84 $\times 10^9$ units, an enrichment of 2.5 times.

Acetone-Water Partition—The acetone-water filtrate was then saturated with NaCl and the mixture allowed to separate into acetone and water layers.   The water layer was shaken with two additional washes of acetone and these were combined with the original acetone layer.   The acetone solution thus obtained contained 53 gm. of solid material with 1.834 $\times 10^9$ units.

Extraction with Petroleum Ether—The acetone solution was boiled down and the residue suspended in 2 liters of water.   This was transferred to a continuous extractor and extracted exhaustively (2 days) with petroleum ether (boiling range 40–60°).   The activity remained quantitatively in the water layer, whose solid content was reduced to 48 gm.

Extraction with Ethyl Ether—The water suspension was then exhaustively extracted with ethyl ether for a week.   (The ether was purified by standing over $CaCl_2$ for a week, filtered, and allowed to stand over metallic sodium from which it was distilled.   Ether obtained by other methods of purification caused a great loss of activity.)   This ether extract contained 4.5 gm. of solid material and activity of 1.83 $\times 10^9$ units.

Acid Wash—The ether extract (2 liters) was then washed with six 100 cc. portions of 5 per cent HCl followed by two 100 cc. portions of water.   400 mg. of completely inactive material were thus removed, the active material remaining in the ether.

Alkali Wash—The ether extract was then washed with six 100 cc. portions of 5 per cent KOH.   Again the activity remained in the ether fraction. The ether was boiled off and the reddish residue weighed 554 mg. and had activity of 1.83 $\times 10^9$ units.   The active material in this residue was then 700 times more concentrated than in the original material.

Solution in and Precipitation from Toluene—The residue was then heated on a water bath at 90–100° with three 10 cc. portions of toluene.   Practically all of the active material was soluble.   The toluene solution was concentrated to about half its original volume and then placed in an ice-salt bath at 0°.   A reddish yellow gelatinous precipitate weighing 113 mg. with activity of 1.7 $\times 10^9$ units was obtained.   At this time the active material had been enriched more than 3000 times.

Phosphotungstic Acid Precipitation—The precipitate from cold toluene

was then taken up in 5 cc. of purified ethyl alcohol.   (The alcohol used was refluxed with powdered zinc for 10 hours, distilled, refluxed with CaO for 12 hours, and again distilled.   Alcohol obtained by other treatments caused loss of activity.)   To the alcoholic solution were then added 5 cc. of a 20 per cent solution of phosphotungstic acid in alcohol.   To this were added 20 cc. of 5 per cent sulfuric acid in water, and the mixture kept at 0° for 24 hours.   The precipitate was removed by centrifugation, dissolved in alcohol, and reprecipitated.   Materials in the combined filtrates and in the precipitate were then recovered by the methods described by Kögl and Tönnis (1936).   The filtrate contained 63 mg. of material with activity of 1.625×10^9 units.   The active substance had now been concentrated more than 5000 times.

Adsorption on Aluminum Oxide—After the solvent was removed from the filtrate, the residue was dissolved in 10 cc. of chloroform.   To this was added 1 gm. of finely pulverized aluminum oxide which had been heated strongly for 5 hours immediately before use.   The flask containing the chloroform solution and the alumina was kept in constant motion for 12 hours.   At this time the aluminum oxide was filtered off.   The chloroform filtrate contained no activity.   The alumina was then shaken with three 10 cc. portions of acetone.   13 mg. of material were recovered from the adsorbing agent in the acetone and its activity assayed at 1.57×10^9 units. Additional elution with acetone-water-ammonium hydroxide yielded 45 mg. of inert substances.   The residue of the acetone eluate contained the active material enriched about 25,000 times.

Precipitation from Chloroform and Ether—The yellowish residue was dissolved in 0.2 cc. of chloroform.   To this was added 0.8 cc. of a mixture of ethyl ether and petroleum ether.   The solution was then placed in an alcohol-carbon dioxide bath at −80° for 6 hours.   The solvent mixture was then filtered off, leaving 4.2 mg. of a creamy white flocculent precipitate. In this particular case approximately equal amounts of activity, 7×10^8 units, went into the two fractions.   The greater part of the activity in the filtrate was recovered by repetition of the precipitation, but subsequent precipitates contained a much higher percentage of inert material.   At other times a much higher percentage of the active material had been precipitated out in the initial chilling.   Even so the concentration of active material in the precipitate was 35,000 times that in the original material.

Solution in Hot Water— The active substance was then dissolved in three 1 cc. portions of boiling water.   After the water was boiled off, the filtrate of partially crystallized creamy white material weighed approximately 2 mg. with activity of 7×10^8 units.

The active material in this residue had been enriched slightly more than 70,000 times as compared with the dry weight of the starting material and

the residue caused the male test plant to produce antheridial hyphae in the concentration of 10^{-12}.

Crystallization from water and from toluene yielded two crystalline substances (m.p. 155°, 218°) both of which were inactive, the activity in both cases remaining in the mother liquor.

SUMMARY

1. The hormonal coordinating mechanism of the sexual process in *Achlya* is briefly reviewed.

2. A technique is described for culturing the female plant of *Achlya bisexualis* in sufficient quantity to furnish material for the chemical study of hormone A.

3. A modification of the biological assay for hormone A is described.

4. Many of the properties of hormone A have been determined: (a) solubilities in common organic solvents, (b) adsorption, (c) stability, (d) inactivation, and (e) reactions with certain reagents.

5. A procedure is described whereby enormous enrichment of the active principle has been achieved.

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PANTOTHENIC ACID DEFICIENCY STUDIES IN DOGS*

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The importance of pantothenic acid in the nutrition of the dog was suggested by early studies in this laboratory (1), by Morgan and Simms (2), and by Fouts *et al.* (3). In all of these studies highly purified rations were supplemented with thiamine, riboflavin, nicotinic acid, and pyridoxine or concentrates of pyridoxine. Since these rations would permit survival only when supplemented with liver or yeast extracts, the general conclusion was reached that a "filtrate factor" was required in addition to the first four synthetic vitamins. An attempt to identify this factor with pantothenic acid was made in this laboratory (4) by treating active liver extracts with alkali or extracting them with acid ether. Deficiencies produced by rations thus supplemented were remedied by highly purified pantothenic acid concentrates from liver extract. Since the availability of synthetic pantothenic acid, no reports have been made on responses of dogs to the pure vitamin. In this paper we wish to describe pantothenic acid deficiency as seen in this laboratory, the response to synthetic pantothenic acid, and some preliminary results on the biochemistry of pantothenic acid deficiency.

EXPERIMENTAL

Weanling mongrel puppies and older growing dogs were used in these studies. The basal sucrose-casein ration used in this laboratory for all vitamin B complex studies has been described (1). It consists of sucrose 66 per cent, acid-washed casein 19 per cent, cottonseed oil 8 per cent, cod liver oil 3 per cent, and salt mixture 4 per cent. Since earlier studies have shown that dogs may be maintained with good growth on this ration supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline over rather long periods of time (5), it was decided to use such a synthetic supplement mixture without pantothenic

acid. The basal ration was therefore supplemented with 100 γ per kilo of body weight per day of thiamine and riboflavin, 2 mg. of nicotinic acid, 60 γ of pyridoxine hydrochloride, and 50 mg. of choline chloride. The vitamins were given twice weekly in aqueous solution.

Blood samples for analysis were removed from the radial vein. Saturated sodium citrate filled the dead space in the syringe, serving as anticoagulant. The blood sugar, non-protein nitrogen, and chlorides were determined on the Folin and Wu tungstic acid filtrates of the citrated blood. Plasma calcium and inorganic phosphorus were determined on trichloroacetic acid filtrates of the plasma. Blood sugar was determined by the method of Benedict (6), non-protein nitrogen by that of Folin and Wu (7), and chlorides by the method of Whitehorn (8). Calcium was determined by precipitation of calcium as oxalate and titration of the oxalate with standard potassium permanganate. Phosphorus was determined by the method of Fiske and Subbarow (9). All colorimetric determinations (sugar, non-protein nitrogen, and phosphorus) were done with the Evelyn photoelectric colorimeter.

A litter of five weanling puppies and three adult dogs were placed on the basal ration. The animals ate the ration well and grew normally for 3 or 4 weeks; then growth ceased abruptly, although food consumption was not appreciably decreased. Failure was extremely sudden and Dogs 213 and 214 died before the deficiencies could be treated. Accordingly as soon as failing symptoms appeared in the other dogs they were given calcium pantothenate orally or parenterally ranging in dosage from 5 to 20 mg. Growth responses were immediate and the animals were allowed to grow for longer periods of time by the periodic administration of calcium pantothenate. Dogs 211 and 212 were allowed to develop a more severe deficiency before administration of pantothenate and were found in a prostrate condition. Both dogs showed rapid labored respiration (43 and 57 per minute) and a slightly increased heart rate (130 and 132 per minute). There were convulsive kicking movements in Dog 211. Administration of calcium pantothenate intravenously produced no effect and both dogs died. Chemical data from a sample of blood taken at the terminal stage of the deficiency from Dog 211 is given in Table I.

Dog 215, as shown in Fig. 1, reached the same severe state of deficiency with comparable symptoms. Intravenous injection of calcium pantothenate and glucose relieved the acute symptoms but the animal required several more injections of pantothenate over a 10 day period before food consumption and growth returned to normal. Blood chemical data from samples taken during the acute deficiency and after growth was resumed are presented in Table I and will be discussed later.

A comparable set of conditions was obtained with the three adult

dogs. Dog 195, after responding satisfactorily to one administration of calcium pantothenate, grew erratically and died suddenly. Dog 199 responded twice to calcium pantothenate when in a less severe state of deficiency. Later the more severe form was allowed to develop, which was characterized by a deep coma, rapid respiration (66 per minute), and a heart rate too rapid and irregular to count. Injection of calcium pantothenate and glucose intravenously resulted in relief from the coma in

TABLE I

Chemical Changes in Blood of Pantothenic Acid-Deficient Dogs before and after Therapy

Dog No.	Litter	Ca pantothenate per kilo body weight per day	Before therapy						After therapy			
			Character of deficiency	Glucose	Non-protein nitrogen	NaCl	Ca	P	Time interval	Glucose	Non-protein nitrogen	NaCl
		γ		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent
211	C	0	Severe	15	61.6		10.1	8.8	Died			
215	"	0	"	48	104.0		10.9	10.3	24 days	80	49.8	477
221	D	0	"	63	95.7				20 "	89	48.0	483
220	"	20	"	20	145.4		9.8	13.7	Died			
219	"	40	Mild	93	46.4		12.6	9.1				
219	"	40	Severe	65	61.7	484			7 days	116	47.4	488
218	"	60	"	81	68.9		12.0	10.0	46 "	102.4	52.2	463
217	"	100	Normal	119	46.2		13.4	9.9				
217	"	100	"	87	44.6	449						
224	E	150	"	102	46.7	454						
225	"	100	"	105	45.1	500						
230	"	40	Mild	53	65.5	481						
230	"	40	Severe	45	115.6	430						
227	"	20	Mild	107	53.3	495						
227	"	20	Severe	62	104.5	398			Died			
228	"	0	Mild	97	51.5	481						
228	"	0	" to severe	131	106.0	416						
229	"	0	Mild	122	44.4	484						
199	F	0	"	64	60.3	346						
199	"	0	Severe	181	93.3	384			18 days	116	47.4	488

2½ hours. The animal did not show improved appetite and on the 3rd day liver extract powder 1:20 (The Wilson Laboratories) was given and continued to a total dosage of 50 gm. in a period of 5 days. Response was very slow and almost 2 weeks were required before the animal could begin to recover the weight lost (Fig. 1).

Dog 193 was allowed to develop the severe deficiency which was characterized by a comatose state but differed from that of Dog 199 in that the

respiratory rate was very slow (14 per minute) and the heart rate was only slightly above normal (135 per minute). There were frequent convulsive
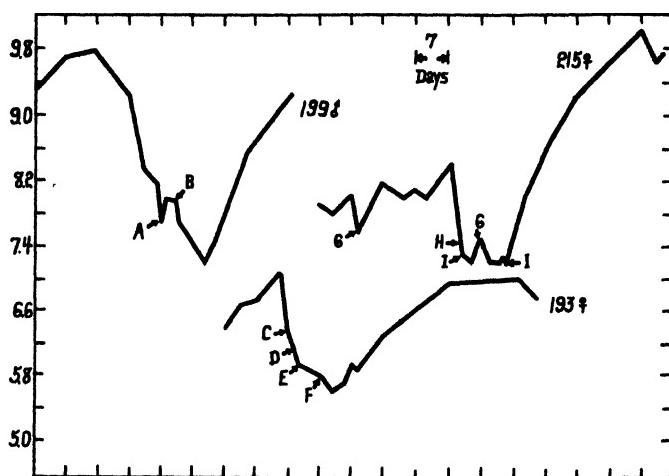
FIG. 1. Response of three dogs on a pantothenic acid-deficient ration. *Dog 199*, A = 20 mg. of calcium pantothenate plus glucose injected intravenously; B = started feeding orally 50 gm. "1:20 liver extract powder" (The Wilson Laboratories) over a 5 day period. *Dog 193*, C = 40 mg. of calcium pantothenate injected intravenously plus sucrose orally; D = 30 mg. of calcium pantothenate injected intravenously; E = 20 mg. of calcium pantothenate injected intravenously; F = started feeding orally 60 gm. "1:20 liver extract powder" over a 5 day period. *Dog. 215*, G = 10 mg. of calcium pantothenate injected intravenously; H = 10 mg. of calcium pantothenate plus glucose in saline solution injected intravenously; I = 5 mg. of calcium pantothenate injected intravenously.

TABLE II

Fat Analysis on Livers of Pantothenic Acid-Deficient Dogs

Dog No.	Litter.	Per cent fat on dry basis
Normal		13–17
195	A	34.7
208	B	46.2
211	C	55.1
212	"	44.3
213	"	43.1
214	"	42.0
220	D	51.6

movements.   Intravenous injection of calcium pantothenate in saline and oral administration of sucrose produced recovery from this severely

deficient condition in 36 hours. Repeated injections of pantothenate over a 7 day period failed to bring the dog back on food. Liver extract powder 1:20 was then given to a total dosage of 60 gm. in a 4 day period. The dog then went back on food and regained weight as shown in Fig. 1.

Necropsies were performed on all the dogs immediately after death. All animals had light colored, mottled livers extremely high in fat content. The fat content, as determined by 24 hour continuous chloroform extraction on the dry material in Caldwell extractors, is given in Table II. Five of the six dogs showed mottled thymus glands with red pigmented spots
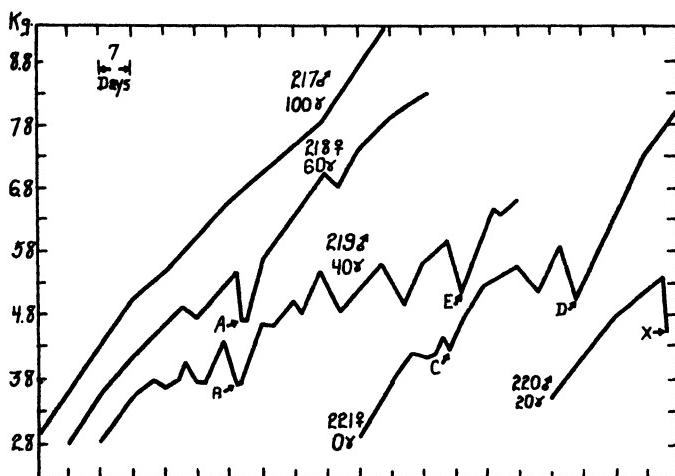
FIG. 2. Growth of five litter mate puppies on various levels of calcium pantothenate. The levels fed per kilo of body weight per day are indicated on the graph. A, 10 mg. of calcium pantothenate plus glucose in saline injected intravenously; B, 12.5 mg. of calcium pantothenate plus glucose injected intravenously; C, 5 mg. of calcium pantothenate injected subcutaneously; D, 15 mg. of calcium pantothenate plus glucose in saline injected intravenously; E, 10 mg. of calcium pantothenate plus glucose injected intravenously; X, died.

suggestive of hemorrhagic degeneration. In most cases the glands were enlarged. The kidneys were dark red in color, showing the stellate veins as distended and cyanotic. There was macroscopic evidence of hemorrhagic degeneration in the cortex and medulla. There was usually a mild gastritis in the pyloric region of the stomach. Four of the six dogs showed slight to severe enteritis. In Dog 195 an intussusception was found, in which case the duodenum at the region of the cap was invaginated into the pylorus. The other organs in the deficient dogs appeared normal.

A litter of five dogs (Nos. 217 to 221 inclusive) was then placed on the ration supplemented as follows: Dog 221 received the basal ration alone,

Dog 220 received 20 γ per kilo of body weight per day of calcium pantothenate, Dog 219 received 40 γ, Dog 218 received 60 γ, and Dog 217 received 100 γ. The growth of these dogs is shown in Fig. 2. Dog 217 showed excellent growth over the 11 week period. Dog 218, receiving 60 γ per kilo of calcium pantothenate, became severely deficient after about 38 days. The symptoms included a respiratory rate of 120 per minute and a heart rate of 140 per minute. Injection of glucose in saline and calcium pantothenate intravenously resulted in immediate recovery and growth was resumed. Dog 219, receiving 40 γ per kilo of calcium pantothenate, developed a severe deficiency in 4 weeks characterized by
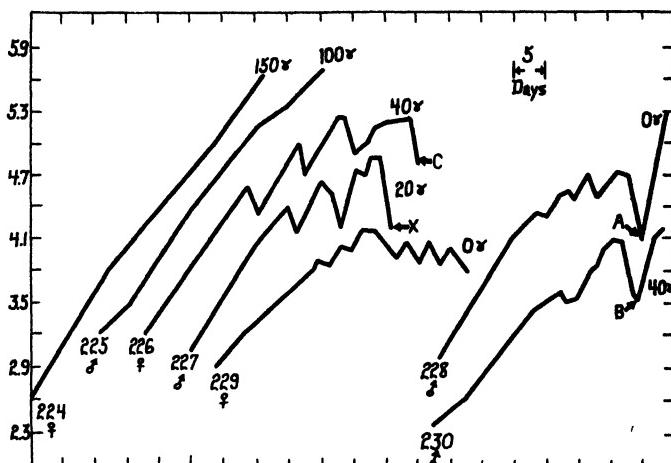
FIG. 3. Growth of seven litter mate puppies on various levels of calcium pantothenate. The levels fed per kilo of body weight per day are indicated on the graph. A, 10 mg. of calcium pantothenate injected intravenously; B, 10 mg. of calcium pantothenate plus glucose injected intravenously; X, 10 mg. of calcium pantothenate plus glucose injected intravenously. Died 8 hours after therapy; C, died.

extreme prostration. Injection of calcium pantothenate in saline intravenously resulted in immediate recovery and resumption of growth. A growth plateau then occurred and in 7 weeks another severe deficiency resulted. An injection of 10 mg. of calcium pantothenate and 6 gm. of glucose intravenously produced an immediate remission.

Dog 220 grew well for 26 days, then rapid failure occurred, and death ensued without the animal going off food. Failure was characterized by vomiting, rapid heart rate, and deep labored breathing. Necropsy revealed three intussusceptions in the lower ileum, and the kidney and liver gave the same appearance as those described above. The other organs appeared normal. Blood findings at the terminal stage are given in Table I.

Dog 221, receiving no pantothenate, developed a mild deficiency in 20 days, which was corrected by subcutaneous injection of 5 mg. of calcium pantothenate. 4 weeks later a severe deficiency developed, showing the same rapid respiratory and heart rates. Intravenous injection of 15 mg. of calcium pantothenate and 6.25 gm. of glucose in saline resulted in an immediate recovery.

Another litter of seven puppies was placed on the basal ration. Dogs 228 and 229 received the basal ration unsupplemented. Dog 227 received 20 γ of calcium pantothenate per kilo of body weight per day, Dogs 226 and 230 received 40 γ, Dog 225 received 100 γ, and Dog 224 received 150 γ. The response of these dogs is shown in Fig. 3. Dogs 224 and 225 grew without developing a deficiency. Dog 230, receiving 40 γ, developed a severe deficiency manifested by extreme prostration. Blood samples taken as the deficiency appeared showed very rapid changes within 14 hours, as shown in Table I. Intravenous injection of 10 mg. of calcium pantothenate and 6 gm. of glucose resulted in the disappearance of symptoms and resumption of growth.

Dog 227, receiving 20 γ of pantothenate, developed a severe deficiency in 31 days. Blood sugar, non-protein nitrogen, and chloride showed very rapid changes in the last 21 hours of this deficiency, as shown in Table I. Death occurred in spite of 10 mg. of calcium pantothenate and 6 gm. of glucose injected intravenously. The thymus, liver, kidney, and stomach showed the characteristic changes previously described. The other organs were normal.

Dog 228, receiving no pantothenate, became deficient and showed a growth plateau in 31 days. The acute symptoms were not allowed to develop and intravenous injection of 10 mg. of calcium pantothenate produced an immediate recovery.

Dog 226, receiving 40 γ, grew fairly well for 6 weeks and then died suddenly. A necropsy revealed the usual changes in the liver, thymus, and kidney and a severe intussusception in which the duodenal cap was invaginated back into the pyloric region of the stomach.

DISCUSSION

From these results it is evident that pantothenic acid deficiency in young growing puppies and adult dogs can be produced on rations of this type. The requirement for the vitamin would seem to be approximately 100 γ of the calcium salt per kilo of body weight per day for the young growing puppies and considerably less than this amount for adult dogs. The deficiency manifests itself very suddenly and the dogs must be observed frequently in order that treatment may be administered in time to save the life of the animal. The deficient dog will eat the ration and grow at a

normal rate usually until the day before the acute deficiency occurs, and sometimes food consumption is normal until the day of the acute onset. Frequently, however, food consumption will be normal and growth will cease several days before the acute symptoms appear. This is the only advance indication of the deficiency. The distressing cardiac, respiratory, intestinal, and skeletal muscular symptoms may be due to nervous lesions or may be brought on by certain changes in the tissues and blood. The fatal character of the deficiency is easily understandable when one considers the severity of the liver pathology and perhaps that of the kidney, thymus, and gastrointestinal tract.

From the chemical data for blood given in Table I, it seems apparent that the plasma calcium and inorganic phosphorus remained essentially normal during pantothenic acid deficiency. The blood glucose was usually significantly lowered during the deficiency and returned to normal upon remission. The glucose levels in the deficient dogs were variable but in general the severely deficient dogs showed the lowest values. The comatose state of a number of dogs may be explained on the basis of the extreme hypoglycemia. The hypoglycemia is of interest in connection with the severe fatty degeneration of the liver which suggests an absence of liver glycogen. Such a condition arising from the ingestion of a ration containing 66 per cent sucrose might suggest a fundamental impairment of carbohydrate metabolism.

The non-protein nitrogen of the blood of the deficient animals was raised significantly over that of the controls. Remission with pantothenic acid therapy reduced these values to normal. The blood chlorides in most cases were 20 per cent lower in the severely deficient dogs than in the controls. This also returned to normal with pantothenic acid administration.

These results are at some variance with those reported by Fouts et al. (3). These observers found fatty livers in their "factor II"-deficient dogs but the total blood chlorides, urea nitrogen, and glucose values in the deficient dogs did not show any significant changes from the normal. The authors concluded from these studies that adrenal cortical insufficiency was not involved. In comparing our results with theirs it must be emphasized that the composition of the rations used in the two laboratories differs, and therefore the two studies are not strictly comparable. Certainly we have observed no macroscopically visible changes in the adrenal glands of any of the deficient dogs, save a slight enlargement noticed in one case.

However, an important point to consider in the study of the blood changes is the rapidity of change in the terminal 24 hours, as shown by Dogs 227 and 230. In this connection the data of Fouts et al. (3) given for one dog in which a sample of blood was taken at the terminal stages of the

deficiency show a drop in the chlorides and a striking increase in urea nitrogen. On the basis of the evidence given above and mindful of the nature of the deficiency in the rat (10) we feel that the question of adrenal insufficiency should be reopened for further experimental work. Hence the failure of Dog 227 to respond to pantothenate and glucose might suggest the additional use of sodium chloride in the treatment of the severely deficient dog.

The failure of Dog 193 to respond immediately to calcium pantothenate and the subsequent response to liver extract powder might suggest that a multiple deficiency exists. However, Dog 199 also failed to respond to pantothenate and the liver extract together until a period of nearly 2 weeks had elapsed, and Dog 215 showed a similar delayed response but was finally brought back on food by pantothenate alone. In all these cases a period of 10 days to 2 weeks was necessary before the dogs started to eat their food and thus gain weight. The results suggest that a multiple deficiency is not involved, but rather that considerable repair of tissue damage must be accomplished before a normal state can be reached. The extensive liver damage seen in all of the dogs dead of the acute deficiency makes this explanation tenable.

The mottled thymuses observed in this laboratory (1) and by Morgan and Simms in both dogs and foxes (2, 11) seem to be confirmed as due to pantothenic acid deficiency. The suggestion of the latter authors of the rôle of the "filtrate factor" in status thymicolymphaticus is an extremely interesting one in view of the short time necessary for fatal pantothenic acid deficiency to occur. It is of interest that regression of the thymus has been observed by Griffith and Wade (12) in choline-deficient rats.

Intussusceptions were observed in earlier studies (4) and the incidence reported here indicates that they are due to pantothenic acid deficiency. In some deficient dogs in which no intussusception was found at necropsy we have observed vomiting attacks so severe that fecal material appeared in the vomitus. There is, then, a fairly consistent severe gastrointestinal phase of this deficiency. The possibility of a nutritional background for intussusception in human infants is suggested by these studies.

The relation of the "filtrate factor" to pigmentation of the hair in dogs was suggested by Morgan and Simms (2) after observing graying on their pantothenic acid-deficient ration. We have observed graying in a number of dogs on our ration supplemented with 500 γ of calcium pantothenate per kilo of body weight per day. This is about 5 times the amount necessary for good growth on this ration and would seem to eliminate pantothenic acid as the responsible factor. Graying of the hair is frequently observed in dogs receiving rations of this type and if it has a nutritional origin, the nature of the factor or factors involved is unknown.

The appearance of the kidneys of the deficient dogs is very similar to that described by Griffith and Wade in choline-deficient rats (12) but is perhaps less severe. The amount of choline supplemented to this ration should rule out choline deficiency as etiologic for this condition. Moreover, dogs receiving adequate pantothenic acid and the same amount of choline do not show these kidney changes.

SUMMARY

1. The requirement of the dog for pantothenic acid is confirmed. The quantitative requirement for young growing puppies is tentatively fixed at approximately 100 γ of calcium pantothenate per kilo of body weight per day.

2. Pantothenic acid deficiency in dogs is characterized by sudden prostration or coma, usually rapid respiratory and heart rate, convulsions, and gastrointestinal symptoms. Necropsy of pantothenic acid-deficient dogs reveals fatty livers, mottled thymuses, evidence of hemorrhagic kidney degeneration, and frequently gastritis or enteritis and intussusception.

3. The plasma calcium and inorganic phosphorus are normal in the deficient dogs, while the blood glucose and chlorides are lowered and the nonprotein nitrogen values are raised. These values return to normal after recovery from the deficiency.

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THE NORMAL RATE OF REDUCTION OF METHEMOGLOBIN IN DOGS*

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Methemoglobin is not present in spectroscopically detectable amounts[1] in freshly drawn blood of normal animals of the following and, presumably, other mammalian species: human, dog, cat, rabbit, rat, mouse, horse, monkey (*Macacus rhesus*), cattle, swine. Furthermore, methemoglobin is not demonstrable in incubated sterile blood from normal animals of these species for many hours after removal from the body. The lag in accumulation of methemoglobin in drawn blood was shown by Warburg, Kubowitz, and Christian (4) to be due to intrinsic enzyme systems of the erythrocytes which reduce methemoglobin to hemoglobin. These workers found that the reducing agents acting *in vitro* are formed primarily from glucose by the erythrocytes. Lactic acid, activated by an intraerythrocytic enzyme system, accounts for 25 to 50 per cent of the reduction (5). Other reductants have not been identified.

One objective of the experiments reported in this paper was to determine the extent to which the enzyme systems contained within the blood account for *in vivo* reduction of methemoglobin and, thus, for maintenance of the circulating hemoglobin in a functionally active form. Before this was undertaken, it was considered advisable to ascertain the normal physiological rate of methemoglobin reduction *in vivo* and the influence upon this rate of several physical and chemical factors. Incident to these studies we have determined the course of methemoglobin accumulation and disappearance following administration of a number of recognized methemo-

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* Some of the data reported in this paper are taken from a thesis presented by one of the authors (W. W. C.) to the Graduate Committee of the University of Tennessee in partial fulfilment of the requirements for the degree of Master of Science, September, 1939. A preliminary report has appeared (1).

[1] Recently several groups of workers (2, 3), using photoelectric, colorimetric methods or the carbon monoxide capacity method for methemoglobin estimation, have reported that samples of blood from normal animals of some of the above species may contain up to 15 per cent methemoglobin. Such quantities would be readily detectable by qualitative spectroscopic examination, which is sensitive to as little as 4 per cent of the total pigment. The common misstatement that methemoglobin is detectable spectroscopically only when its concentration is 15 to 20 per cent or more of the total pigment has been repeated in a recent paper by Ammundsen (3).

globin-forming agents and several not hitherto tested. Also, the catalytic action of methylene blue in hastening reduction of methemoglobin to hemoglobin has been further explored.

Methods

Dogs which had been fasted for 18 hours or longer were used as experimental animals. If the administered substance was readily soluble in water, it was injected intravenously; if only sparingly soluble, it was suspended in olive oil, or mineral oil, and given by stomach tube.

In most instances methemoglobin was determined by a rapid spectroscopic method (6) which is referred to in this paper as the *dilution method*. Since this method is relatively new, we have compared values obtained by it with those given by the spectrophotometric method of Austin and Drabkin (7), and also with values given by the method of difference between total pigment and oxygen capacity (8). Total pigment was determined spectrophotometrically as cyanmethemoglobin (7). Comparative analyses of forty samples of blood containing methemoglobin due to thirteen different substances have shown that the method of difference between total pigment and oxygen capacity gives consistently the lowest methemoglobin concentrations. Values obtained by the Austin and Drabkin method average 4 per cent higher, and those by the dilution method 9 per cent higher. The divergences, however, are unrelated either to concentration of methemoglobin, which varied between 10 and 60 per cent of the total pigment, or to total pigment, which varied between 7 and 22 volumes per cent. In these comparison studies the analyses were so ordered that the results are not vitiated by *in vitro* changes in methemoglobin concentration in the samples.

Blood glucose was determined by the Shaffer-Hartmann method (9) on zinc filtrates (10).

EXPERIMENTAL

In searching for substances which might be useful in producing methemoglobin, we have tested twenty-five compounds. Single doses of the following substances (the dose, in mg. per kilo, is given after each substance and the mode of administration is indicated by V, intravenous, or T, stomach tube) regularly produced methemoglobin: acetanilide, 200, T; o-aminophenol, 20, V; p-aminophenol, 20, V; aniline, 50, V and T; dimethylaniline, 50, T; hydroxylamine, 5, V; α- or β-naphthylamine, 200, T; p-nitroaniline, 15, V; nitrobenzene, 200, T; nitroglycerin, 10, V; sodium dichromate, 60, V; sodium nitrite, 30, V. The following substances produced methemoglobin only after several daily administrations of large doses: bismuth subnitrate, 1000, T; plasmochin, 1, T; and promin, 600, T.

Single doses of H acid (30, V), hydroquinone (30, V), o-nitrophenol (700, T), and p-nitrotoluene (50, T) caused no accumulation of methemoglobin. Dinitrophenol, sodium chlorate, sodium ferricyanide, sodium sulfanilate, and sulfanilamide gave negative results even after repeated administrations of large doses.

The course of accumulation of methemoglobin in the blood and its subsequent disappearance following administration of single doses of some of these substances are illustrated in Fig. 1.   Each curve represents an average result of several experiments.   The rates of accumulation as well as the rates of disappearance of methemoglobin vary greatly.   Thus, intrave-
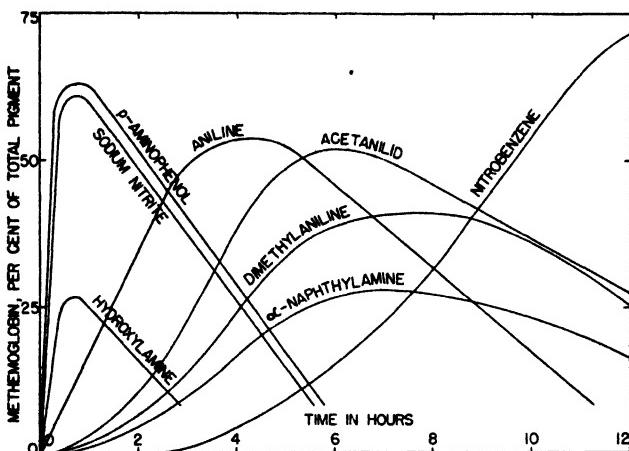
Fig. 1.   Representative variations in the accumulation and disappearance of methemoglobin.   Acetanilide, dimethylaniline, α-naphthylamine, and nitrobenzene were administered orally.   Other substances were injected intravenously.

nously injected nitrite produces a maximal concentration of methemoglobin in about 45 minutes, whereas orally administered nitrobenzene is not maximally effective for 12 to 15 hours.   Also, as others have noted, we have encountered considerable individual variation in the response to oral administration of a given organic methemoglobin-forming compound.   The rate of accumulation, the maximal methemoglobin concentration, and the rate of disappearance all may vary widely following administration of a constant dose of the substance.

In contrast to oral administration of most of these substances, intravenous administration of several of them yields quite reproducible results. Table I summarizes the results of thirty-three experiments in which methemoglobin-forming substances were injected intravenously.   The rates of disappearance of methemoglobin formed by intravenous injection

of o-aminophenol, p-aminophenol, or sodium nitrite proved to be the same
and are the most rapid yet observed. Methemoglobin produced by aniline
or p-nitroaniline, perhaps, disappears more slowly.

As judged by twenty experiments on fourteen dogs, the mean rate of dis-
appearance of methemoglobin following injection of sodium nitrite (0.5
cc. of 6 per cent solution per kilo) is 11.2 per cent of the total pigment per
hour. The maximum and minimum rates were, respectively, 16.1 and
7.3 per cent of the total pigment per hour. The standard deviation of the
mean is ±2.0 per cent of the total pigment per hour. In these experiments
total pigment ranged in different dogs between 22.0 and 6.2 volumes per
cent. However, the rate of methemoglobin disappearance, expressed as
per cent of the total pigment, is independent of the total pigment concen-
tration and the methemoglobin concentration. Thus, in one animal
methemoglobin disappeared at the rate of 12.2 per cent per hour when the

TABLE I

*Rate of Reconversion of Methemoglobin to Hemoglobin in Vivo Following Intravenous
Injection of Methemoglobin-Forming Agents*

Substance injected	Amount injected	No. of experiments	Average time to reach maximal methemoglobin concentration	Rate of methemoglobin disappearance	Standard deviation
	mg. per kg.		*min*	*per cent total pigment per hr.*	*per cent total pigment per hr.*
o-Aminophenol.............	20	3	45	11.2	±3.0
p-Aminophenol............	20	5	45	11.8	±1.4
Aniline.................	50	5	200	9.6	±2.9
Sodium nitrite	30	20	45	11.2	±2.0

total pigment was 16.0 volumes per cent. After this animal was made
anemic by repeated hemorrhage (total pigment = 6.2 volumes per cent),
the rate of disappearance was unchanged.

Means of Altering Rate of Reversion of Methemoglobin to Hemoglobin

Methylene Blue—From observations on humans, dogs, and rabbits it has
been found previously (11–13) that intravenous injection of methylene blue
accelerates reduction of methemoglobin to hemoglobin when the methemo-
globinemia is induced by aniline, nitrite, p-bromoaniline, nitrobenzene,
p-aminophenol, or the sulfonamides. Methemoglobinemia in dogs due to
acetanilide, o-aminophenol, dimethylaniline, α- or β-naphthylamine, o-
or p-nitroaniline, also, we now find to respond to methylene blue injections.

As was pointed out previously, intravenously injected methylene blue
may produce only a temporary reduction of methemoglobin in humans re-
ceiving the sulfonamide drugs (12, 13). The same is true in dogs which

have received large amounts of acetanilide, dimethylaniline, or nitrobenzene orally. Fig. 2 illustrates this behavior and shows also, as compared with the first injection of methylene blue, a diminished effectiveness of two subsequent injections. The latter phenomenon is probably a result of an
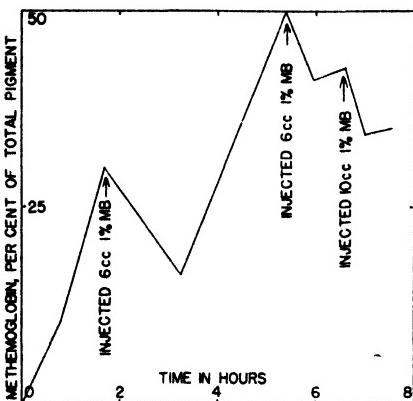
FIG. 2. Decreasing effectiveness of successive injections of methylene blue in catalyzing reduction of methemoglobin. This dog received 0.7 gm. of acetanilide (in mineral oil) per kilo by stomach tube at zero time.
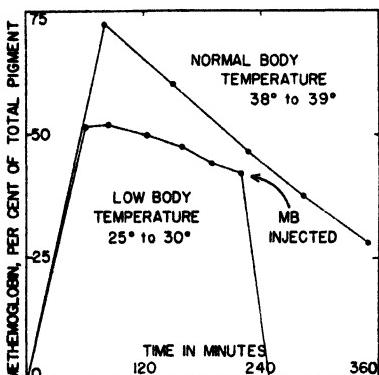
FIG. 3. Effect of body temperature upon the rate of disappearance of methemoglobin. *MB* = methylene blue.

increase in concentration of the active pigment-oxidizing agent, owing to prolonged absorption of the drug.

Temperature—The rate of disappearance of methemoglobin in one dog with normal body temperature was found to be 10.3 per cent of the total pigment per hour (Fig. 3). When the animal's body temperature was lowered about 10° by packing in ice, the rate decreased to 3.3 per cent per

hour.   Methylene blue was effective, however, in accelerating reconversion of methemoglobin to hemoglobin at the lowered body temperature.   That the slowness of disappearance of methemoglobin at lowered body temperature is not due to prolonged methemoglobin formation is indicated by the fact that a sample of blood drawn 1 hour after injection of nitrite was free of nitrite, as judged by the starch-iodide test.   Three other animals gave similar results.   Nembutal was used to maintain light anesthesia in these experiments.

TABLE II

Comparison of Rates of Disappearance of Methemoglobin in Vivo and in Vitro and Effect of Blood Sugar Concentration in Vitro

Dog No.		Blood glucose concentration at 0 time	Methemoglobin concentration at intervals after maximum concentration is reached, per cent total pigment									Average rate of disappearance of methemoglobin
			0* min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.	
		mg. per cent										*per cent total pigment per hr.*
1	*In vivo*	93	54	49	44	41	37	33	29	25		8
	" *vitro*	93	54	49	45	41	38	33	29	25		8
	" " + glucose	293	54	50	46	43	39	35	30	27		8
2	" *vivo*	293	76		67	60	51	46	41	36	31	11
	" *vitro*	293	76		67	61	54	48	43	39	35	10
	" " + glucose	493	76		67	60	52	45	42	38	35	10

* Time of maximum methemoglobin concentration.

Factors Which Do Not Affect Rate of Reversion of Methemoglobin to Hemoglobin

Blood Sugar Concentration—Dog 1 (Table II) was given sodium nitrite intravenously.   At the end of an hour 50 cc. of blood were drawn, defibrinated, and divided equally between two flasks.   50 mg. of glucose were added to the blood in one of the flasks and the two samples were then incubated at 37.5° with gentle rocking.   The concentration of methemoglobin was determined from time to time in the incubated samples as well as in further samples drawn from the dog.   The same procedure was followed in Dog 2, except that this animal received 5 gm. of glucose per kilo orally before nitrite was injected.   Results of these two experiments show that at comparable temperatures the *in vivo* and *in vitro* rates of disappearance of methemoglobin are the same, and that elevation of blood sugar *in vitro* has no effect upon the rate during the period required for conversion of 50 per cent of the methemoglobin to hemoglobin.

The experiments summarized in Table III show a lack of effect of increased blood sugar concentration upon the rate of disappearance of methemoglobin *in vivo*. In Experiments 1 and 2 the rate was determined while the animal's blood sugar was at normal levels. In Experiments 3 and 4

TABLE III

Lack of Effect of Glucose Concentration on Rate of Methemoglobin Disappearance in Vivo

Experiment No.	Blood glucose concentration at 0 time	Methemoglobin concentration at intervals after maximum concentration is reached, per cent total pigment							Average rate of methemoglobin disappearance
		0 min.*	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	
	mg. per cent								*per cent total pigment per hr.*
1	Normal†	54	47	40	33	26	20		14
2	"	55	50	42	34	27			14
3	120	53	45	36	31	26			14
4	245	66	60	53	47	41	34	27	13

* Time of maximum methemoglobin concentration.
† Not determined but found to be normal on other occasions.
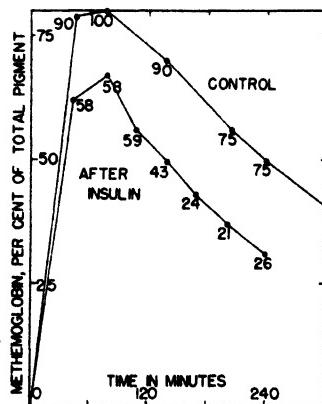
FIG. 4. Lack of influence of low blood sugar concentrations upon rate of disappearance of methemoglobin. The figures below the points are blood glucose concentrations in mg. per cent.

the rate was determined while the blood sugar was elevated by oral administration of glucose.

To test the possible effect of hypoglycemia upon the rate of disappearance of methemoglobin, a dog was given an injection of 30 units of insulin at the same time that nitrite was injected. Results of this experiment are illustrated in Fig. 4, where the connected points represent the methemoglobin concentration at various intervals after injection of nitrite and the

figures below the points are the blood glucose concentrations at the indicated times. The rate of methemoglobin disappearance is not significantly influenced by the lowered blood sugar.

Prolonged Methemoglobinemia—In order to determine the effect of protracted methemoglobinemia upon the ability of blood to reduce methemoglobin, a dog was given intravenous injections of sodium nitrite (30 mg. per kilo) at intervals of 5 to 6 hours for 36 hours. Following the first injection, subsequent injections were made when the methemoglobin concentration had fallen to about 20 per cent. The rate of disappearance was determined after each of the seven injections. The data in Table IV indicate the lack of effect of continuously high concentration of methemoglobin upon the rate of disappearance. It is to be noted that at the average rate of disappearance shown by this animal all of the blood pigment must have under-

TABLE IV

Effect of Successive Injections of Sodium Nitrite on Rate of Methemoglobin Disappearance in Vivo

Nitrite injection	Time since first injection	Maximum methemoglobin concentration	Rate of disappearance of methemoglobin following injection
	min.	*per cent total pigment*	*per cent total pigment per hr.*
1st	0	65	10
2nd	345	63	10
3rd	675	61	11
4th	975	64	10
5th	1305	61	11
6th	1560	52	9
7th	1835	62	11

gone oxidation and reduction at least four times in the period of 36 hours during which methemoglobin was constantly present in the blood.

Fasting—Two animals which were fasted for 3 weeks showed no change in ability to convert methemoglobin to hemoglobin.

DISCUSSION

The average rate at which accumulated methemoglobin within the circulating erythrocytes of dogs is replaced by functionally active hemoglobin is 11.3 ± 2.0 per cent of the total pigment per hour. This rate, observed following injection of such different chemical substances as sodium nitrite, *o*-aminophenol, and *p*-aminophenol, would appear to be the true physiological rate of this vital reduction reaction. The slower rate of disappearance and consequent apparent slower rate of reduction seen when certain other substances are administered are probably explained by pro-

longed formation of methemoglobin, which masks the reduction reaction. In the case of nitrite, methemoglobin formation is rapid and nitrite is equally rapidly destroyed. 60 minutes after intravenous injection of 30 mg. of sodium nitrite per kilo, methemoglobin formation ceases and nitrite is no longer detectable in the plasma by the very sensitive starch-iodide test.

The rate of reduction of methemoglobin is independent of the following factors: (1) methemoglobin concentration, at least when this is greater than 20 per cent of the total pigment; (2) total pigment, *i.e.* hemoglobin plus methemoglobin; and (3) glucose concentration, between 40 and 400 mg. per cent. The rate is not influenced by several weeks of fasting and is sustained *in vivo* for a period of at least 36 hours, during which time all of the pigment in the circulating blood passes through methemoglobin at least four times. Since the rate of reduction *in vivo* is the same as that *in vitro*, the influence of other body tissues appears to be negligible. The rate of reduction is' retarded by low body temperature, but is uninfluenced by 2 or 3 degrees of fever produced by injection of dinitrophenol.

The recognized rôle of glucose as the principal *source* of reducing agents for methemoglobin *in vitro* led Brooks (14) to test the value of intravenous injection of glucose in hastening reduction of methemoglobin *in vivo*. On the basis of experiments with rabbits, injected with nitrite to produce methemoglobin, Brooks concluded that intravenous administration of 10 to 20 mg. of glucose per kilo (Brooks gave 1 to 2 cc. of 1 per cent solution per kilo) greatly accelerates the reconversion of methemoglobin to hemoglobin. In consequence Brooks (15) has recommended intravenous administration of glucose in treatment of human methemoglobinemia. Our experiments on dogs, to which we gave amounts of glucose which produced significant and measured increases in blood sugar concentration, failed to demonstrate any accelerating action of glucose in this species.

The rate of methemoglobin reduction shows no correlation with total pigment only *when the rate is expressed as per cent of total pigment* per unit time. When the rate is expressed in absolute units, *e.g.* volumes per cent per hour, there is a significant decrease in the rate as the total pigment decreases. Thus, one dog before hemorrhage, when the total pigment was 16.0 volumes per cent, showed a rate of reduction of 12.2 per cent of total pigment per hour or 2.0 volumes per cent per hour. After hemorrhage, when the total pigment was 6.2 volumes per cent, the rate expressed as per cent of total pigment was unchanged. Expressed in terms of volumes per cent per hour, the rate had decreased to 0.7.

Finally, the rate of reduction of methemoglobin is much more constant from animal to animal and in a given animal at different times when the percentage mode of expression is employed than when the rate is expressed in absolute units. This is evidenced by the fact that the standard devia-

tion of the mean is approximately twice as great in the latter case as in the former.

There appears to be ample evidence that the maintenance of hemoglobin in a functionally active state is accomplished mainly if not entirely by enzyme systems contained within the circulating erythrocytes.

SUMMARY

Methemoglobin contained within circulating erythrocytes of dogs is reduced to hemoglobin at a constant average rate of 11.3 per cent of the total pigment per hour. This rate, therefore, represents the maximum resistance of this species to accumulation of methemoglobin. Reduction of intracorpuscular methemoglobin is solely a function of enzyme systems contained within the erythrocytes. Ability to reduce methemoglobin is impaired by low body temperature. It is not affected by severe hypoglycemia or by blood sugar concentrations several times the normal. Capacity to convert methemoglobin to hemoglobin is not diminished even after all of the blood pigment has been converted to methemoglobin four times in a relatively short period.

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CYTOCHROME REDUCTASE

II. IMPROVED METHOD OF ISOLATION; INHIBITION AND INACTIVATION; REACTION WITH OXYGEN

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A previous paper (1) reported the isolation of an enzyme, cytochrome c reductase, which can be rapidly reduced and oxidized by dihydrotriphosphopyridine nucleotide and ferricytochrome c, respectively. The reductase thus establishes a link in the chain of respiratory enzymes. The preparations of the enzyme obtained previously contained a small amount of impurities, especially a few per cent of a hemin compound;[1] moreover, there was a rather low yield in some of the isolation steps. Modifications of the previously described procedure increased the yield 8-fold and resulted in a 98 per cent pure reductase, free of hemin.

Isolation of Enzyme

Yeast was washed, dried, and autolyzed, as previously described (1). Table I indicates the amount of enzyme which could be extracted, under optimum conditions, from five different sources. The figures of Table I represent minimum values of the enzyme concentration in yeast, since probably only a fraction of the total enzyme present could be extracted. In the method for the isolation of the enzyme (1), Steps 1 to 4 remain unchanged. Directions for the modified steps follow.

Step 5. Adsorption on Aluminum Hydroxide Gel—5 gm. of the enzyme preparation obtained after Step 4 are dissolved in 200 ml. of water and the pH is adjusted to about 9 with 4 ml. of N potassium hydroxide. γ-Aluminum hydroxide gel (2) is added in fractions, until the supernatant solution becomes colorless. Approximately 7 gm. of aluminum hydroxide are required. The enzyme is eluted from the aluminum hydroxide by twice adding 120 ml. of a solution which is 64 per cent saturated with respect to ammonium sulfate and 0.1 N with respect to ammonium hy-

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[1] This hemin compound, in the reduced form, has the α-band at 557 mμ, the Soret band at 420 mμ. The presence of this hemin compound in yeast was confirmed in a recent communication by Bach, Dixon, and Keilin (*Nature*, **149**, 21 (1942)). They isolated a new soluble cytochrome component, b_2, from yeast, which, on spectrometric evidence, seems to be identical with the one previously observed here (1).

droxide. The elution is continued by adding two 160 ml. portions of a solution which is 40 per cent saturated with ammonium sulfate and 0.1 N with respect to ammonium hydroxide. The enzyme is precipitated from the combined eluates by increasing the ammonium sulfate concentration to 65 per cent saturation and adjusting the hydrogen ion concentration to pH 4.5 (560 ml. of eluate + 90 gm. of solid ammonium sulfate + 70 ml. of 2 N acetate buffer, pH 4.5). The enzyme is separated by centrifugation, dissolved in 150 ml. of water, and the solution brought to pH 9 with 5 ml. of N ammonium hydroxide. This solution contains 1200 mg. of protein: $W = 40$; purity $= 0.25$.

Step 6. Adsorption on Calcium Phosphate Gel—Tricalcium phosphate gel (about 17 gm.) is added fractionally to the enzyme solution until the supernatant becomes colorless. After centrifugation the combined pre-

TABLE I

Cytochrome Reductase Extracted from Yeast

Source material	Time of extraction, room temperature	Concentration
	hrs.	*gm. enzyme per kg. dry yeast*
Fleischmann's bakers' yeast.........	24	0.045
Keeley's beer yeast*.............	48	0.40
Drewry's ale yeast†..............................	33	0.60
Westminster ale yeast‡......	48	1.00
Canadian ale yeast§..............................	34	2.60

* Keeley Brewing Company, Chicago.
† Drewry's, Ltd., South Bend, Indiana.
‡ Westminster Brewing Company, Chicago.
§ Canadian Brewers, Ltd., Toronto.

cipitate is washed with 400 ml. of water and the enzyme is eluted with three 200 ml. portions of 0.2 M phosphate, pH 6.1. The enzyme is precipitated from the combined eluate in order to remove phosphate which interferes in the next purification step. To 600 ml. of the eluate, 310 gm. of solid ammonium sulfate are added (saturation $= 80$ per cent), and the solution is centrifuged. The precipitate, which contains the enzyme, is dissolved in 70 ml. of 0.03 N ammonium hydroxide. The solution now contains 340 mg. of protein: $W = 67$; purity $= 0.43$.

Step 7. Adsorption on Aluminum Hydroxide Repeated—Portions of aluminum hydroxide gel are again added to the enzyme solution until the supernatant liquid is colorless (about 3 gm. of aluminum hydroxide required). The enzyme is eluted from the adsorbent by washing three times with 60 ml. of a solution which is 64 per cent saturated with ammonium sulfate and 0.1 N with respect to ammonium hydroxide. In 180 ml. of the

combined eluates, 140 mg. of enzyme are finally obtained; $W = 155$; purity $= 0.98$.

The enzyme is precipitated at pH 4.5 by increasing the ammonium sulfate concentration to 80 per cent saturation and is collected by centrifugation. The precipitate is treated with 0.30 ml. of 2 N ammonium hydroxide to adjust the pH to about 9. By evaporating in a high vacuum the suspension can be frozen and stored at $0°$ over phosphorus pentoxide (as drying agent). *No decrease in activity was observed* after $2\frac{1}{2}$ months of storage.

Reaction with Oxygen

Cytochrome reductase can be reoxidized by cytochrome c (Equation 1) or by atmospheric oxygen, according to Equation 2.

$$CR \cdot H_2 + 2CyFe^{+++} \rightarrow CR + 2CyFe^{++} + 2H^+ \qquad (1)$$
$$CR \cdot H_2 + O_2 \rightarrow CR + H_2O_2 \qquad (2)$$
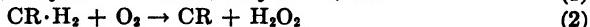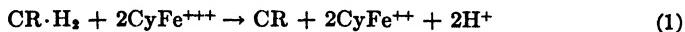
At low oxygen pressure the velocity of Reaction 2 is given by $-dO_2/dt = k_3\,(CR \cdot H_2)\,(O_2)$. The manometric experiment outlined in Table II was performed to determine the reaction rate of cytochrome c reductase with oxygen. A comparison of k_3 (oxygen) with k_2 (ferricytochrome c) shows that cytochrome reductase will react about 7×10^5 times faster with ferricytochrome c than with oxygen. This fact indicates that the direct reaction of the cytochrome reductase with oxygen is very probably without physiological significance.

Comparison with Old Yellow Enzyme—Both the old yellow enzyme of Warburg and Christian (3) and cytochrome reductase have alloxazine mononucleotide as their prosthetic group. However, the two enzymes are not identical with respect to enzymatic activity and other physical properties shown in Table III, in which k_1, k_2, and k_3 represent specific velocity constants for the following reactions.

$$-\frac{d(TPN \cdot H_2)}{dt} = k_1 \text{ (alloxazine)(TPN} \cdot H_2)$$

$$-\frac{d(CyFe^{+++})}{dt} = k_2 \text{ (dihydroalloxazine)(ferricytochrome)}$$

$$-\frac{dO_2}{dt} = k_3 \text{ (dihydroalloxazine)(oxygen)}$$
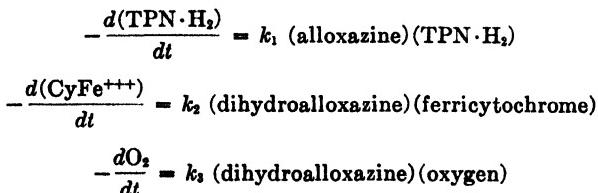
K designates the dissociation constant

$$K = \frac{\text{alloxazine} \times \text{protein}}{\text{enzyme}}$$

Since the prosthetic groups are identical, the protein must be responsible for the different properties of the two enzymes. Replacement of the pro-

TABLE II

Reaction with Oxygen

25°; center cup, 0.1 ml. of 2 N KOH.

	Experiment 1. Air	Experiment 2. Air	Experiment 3. Oxygen
2.25 ml. water............ 0.25 " 0.5 M phosphate, pH 7.1.............. 0.10 ml. 0.025 M HCN..... 0.26 mg. triphosphopyridine nucleotide .... 3.0 mg. *Zwischenferment*.. 10 " glucose-6-phosphate 1.95 × 10⁻⁸ mole cytochrome reductase......	$\longrightarrow$ → 3.90 × 10⁻⁸ mole	$\longrightarrow$ → 1.95 × 10⁻⁸ mole

Oxygen uptake			
min.	*c.mm.*	*c.mm.*	*c.mm.*
10	9.5	19	21
20	19.0	38	42

$$\text{Experiment 1---}v = \frac{dO_2}{dt} = 1.63 \times 10^{-5} \text{ (mole} \times \text{liter}^{-1} \times \text{min.}^{-1})$$

$$(O_2) = 2.7 \times 10^{-4} \text{ (mole} \times \text{liter}^{-1})$$

$$(CR \cdot H_2) = 7.5 \times 10^{-6} \text{ (mole} \times \text{liter}^{-1})$$

$$k_3 = \frac{v}{(O_2)(CR \cdot H_2)} = 0.80 \times 10^4 \text{ (liter} \times \text{min.}^{-1} \times \text{mole}^{-1})$$

TABLE III

Comparison of Cytochrome Reductase and Old Yellow Enzyme

Rate constant k = liter × minute⁻¹ × mole⁻¹; dissociation constant K = liter⁻¹ × mole.

Enzyme	Reaction with			Dissociation constant	Absorption maximum, λ
	Triphosphopyridine nucleotide	Cytochrome *c*	Oxygen		
	k_1*	k_2	k_3	K	$m\mu$
Cytochrome *c* reductase..............	85 × 10⁶	53,000 × 10⁵	0.8 × 10⁴	1 × 10⁻⁹	455
Old yellow enzyme....	6 × 10⁶	0.3 × 10⁵	10 × 10⁴	60 × 10⁻⁹	465

* Details concerning the determination of k_1 and k_2 will be submitted in a communication dealing with the kinetics of the enzymatic reactions.

tein of the old yellow enzyme by that of the reductase increases the activity towards cytochrome and triphosphopyridine nucleotide tremendously but simultaneously diminishes the activity towards oxygen. The autoxi-

dation of free alloxazine mononucleotide is inhibited when it combines with protein and one would therefore expect an enzyme with a small dissociation constant to react slowly with oxygen. The results of Table III confirm this relationship between dissociation of the prosthetic group and reactivity towards atmospheric oxygen.

Denaturation of Cytochrome c Reductase—From the preceding data, it is apparent that the old yellow enzyme is not identical with cytochrome reductase and the question arises whether it could be a degradation product of the cytochrome reductase. In this case the denaturation of the reductase should result in a decreased activity with cytochrome but increased activity with oxygen. We have followed both reactions with enzyme preparations which were denatured by keeping them for several weeks at low temperature or for a few minutes at elevated temperature in 0.025 M phosphate buffer, pH 7.2. The reaction with cytochrome was

TABLE IV

Denaturation of Cytochrome c Reductase

Incubation	Activity towards oxygen	Activity towards cytochrome c
	per cent	*per cent*
0	100	100
28 days at 3°	64*	8.8†
10 min. " 50°	44	8 5
$k = \dfrac{1}{t} \times 2.3 \times \log \dfrac{C_0}{C}$ $*k_{3^\circ} = 1.1 \times 10^{-5}$ (min.$^{-1}$) $k_{50^\circ} = 0.082$ (min.$^{-1}$)		$\dagger k_{3^\circ} = 6.0 \times 10^{-5}$ (min.$^{-1}$) $k_{50^\circ} = 0.246$ (min.$^{-1}$)

measured spectrophotometrically, as described previously (1); the reaction with oxygen, as described in Table II. The results are given in Table IV; k represents the velocity constants for the inactivation of the enzyme towards cytochrome and oxygen, respectively.

The results of Table IV indicate that denaturation of the enzyme diminishes its activity towards both oxygen and cytochrome and therefore the old yellow enzyme cannot be considered as a degradation product of cytochrome reductase. The activity of the enzyme towards cytochrome is destroyed to a greater extent than that towards oxygen. This fact indicates that the mechanism of oxidation by cytochrome differs from that in which oxygen takes part.

Inhibition by Substituted Phenols

Krahl and Clowes (4) and Krahl, Keltch, and Clowes (5), after finding that the respiration of fertilized *Arbacia* eggs is inhibited by certain substituted phenols, showed that with cell-free systems neither the cyto-

chrome oxidase system nor certain dehydrogenase systems are affected by these substances. The catalytic activity of flavoproteins, however, is inhibited.

To obtain information concerning the effect of 2,4-dinitro-*o*-cyclohexyl-phenol on the individual steps involved in the respiratory process, we have investigated its effect on the various isolated components of the system which involves cytochrome *c*, cytochrome reductase, triphosphopyridine nucleotide, *Zwischenferment*, and glucose-6-phosphate. With this information we then tried to correlate the inhibitory effect on isolated systems with that on the respiration of intact bakers' yeast.

Inhibition of Cytochrome Reductase[2]—Cytochrome reductase was incubated with the substituted phenol for 15 minutes (0.001 M phenol, 0.040 M phosphate buffer, pH 8.3, temperature 25°). The enzymatic activity was determined by the specific cytochrome reductase test previously described (1). The result of the experiment showed that under the specified conditions the enzymatic action of cytochrome reductase was 70 per cent inhibited.

Triphosphopyridine Nucleotide (TPN) and Substituted Phenols—The specific test for TPN, described in a previous publication (6), can be used to study the effect of organic compounds on its catalytic activity. Despite incubation in a 0.001 M solution of 2,4-dinitro-*o*-cyclohexylphenol, buffered with phosphate, no effect on the catalytic activity of TPN could be observed. In the activity test the velocity-determining reaction is the oxidation of dihydro-TPN. Therefore one can conclude that dihydro-TPN is not affected by this substituted phenol.

Inhibition of Zwischenferment—The rate of reaction of *Zwischenferment* was measured by ultraviolet spectroscopy in a manner similar to the method of Negelein and Haas (7). The reduction of TPN is indicated by an increase in the light absorption at λ 340 mμ. Under the conditions specified in Table V, the rate of reduction of TPN is a function of the *Zwischenferment* concentration. As the substituted phenols exhibit a strong light absorption in the ultraviolet region, it is necessary to use very thin absorption cells during this test. *Zwischenferment* was incubated for 15 minutes at 25° (0.001 M phenol + 0.05 M phosphate, pH 8.3).

Details concerning the analytical test are given in Table V. The suppression of the reduction of triphosphopyridine nucleotide indicates that 0.001 M 2,4-dinitro-*o*-cyclohexylphenol produces a 90 per cent inhibition of *Zwischenferment*.

Inhibition of Respiration—The action of the substituted phenol on the

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[2] Dr. Krahl suggested this experiment in a private communication and kindly supplied the substituted phenols.

respiration of yeast was determined manometrically; the conditions are given in Table VI. 0.001 M 2,4-dinitro-o-cyclohexylphenol inhibits the respiration of bakers' yeast 93 per cent.

TABLE V

Inhibition of Zwischenferment by 2,4-Dinitro-o-cyclohexylphenol

Wave-length = 340 mμ; length of absorption cell = 0.10 cm.; temperature = 25°.

Experiment 1	Experiment 2
1.0 ml. 0.025 M phosphate buffer, pH 8.3.... 5.8 mg. potassium salt of glucose-6-phosphoric acid..................................... 0.09 mg. *Zwischenferment* (impure)........... 0.36 " triphosphopyridine nucleotide.......	$\longrightarrow$ + 1 × 10^{-3} M phenol

Triphosphopyridine nucleotide reduced in 10 min.	
mg. 0.210	*mg.* 0.032

TABLE VI

Effect of 2,4-Dinitro-o-cyclohexylphenol on Respiration of Bakers' Yeast

Fleischmann's bakers' yeast; temperature, 25°; center cup, 0.1 ml. of 2 N KOH; gas phase, air.

	Experiment 1	Experiment 2	Experiment 3
Side arm	2.3 ml. 0.025 M phosphate, pH 7.2 12 mg. yeast 0 0.2 ml. H$_2$O + 24 mg. glucose (added after 15 min. incubation)	$\longrightarrow$ 12 mg. yeast 1 × 10^{-3} M phenol $\longrightarrow$	$\longrightarrow$ 0 1 × 10^{-3} M phenol $\longrightarrow$

Oxygen uptake			
min.	*c.mm.*	*c.mm.*	*c.mm.*
20	57	5	0
40	118	8	0

A summary of the inhibition experiments with intact yeast and with the isolated systems is presented in Table VII. The components of the isolated system are arranged in the order in which they react. There is no inhibition of the enzyme system which reacts between oxygen and cytochrome *c*. This observation by Krahl and Clowes has been confirmed by

Dr. Bernard Block in our laboratory. Therefore it must be concluded that the interaction of the phenols takes place somewhere in the chain of enzymes between cytochrome *c* and glucose-6-phosphate. The results of Table VII indicate two possible points of interference by substituted phenols in the respiratory system, that is, cytochrome reductase and *Zwischenferment*, only one of which is a flavoprotein. Since the inhibitory action of substituted phenols is not restricted to flavoproteins, no definite conclusion concerning the significance of these enzymes in the respiration of the living cell can be obtained from these inhibition experiments.

TABLE VII

Effect of Substituted Phenols on Isolated Intermediary Steps and on Respiration of Living Cells

Enzyme system		Inhibition by 1×10^{-3} M 2,4-dinitro-*o*-cyclohexyl-phenol
		per cent
Oxygen	}cytochrome oxidase.	0
Cytochrome *c*	}cytochrome reductase .	70
Triphosphopyridine nucleotide	}*Zwischenferment* . . .	90
Glucose-6-phosphate		
Respiration of bakers' yeast..		.93

SUMMARY

1. With an improved procedure of isolation, cytochrome reductase can be obtained with a purity of 98 per cent and with 8-fold better yield than previously reported.

2. Since the enzyme reacts about 10^6 times faster with cytochrome than with molecular oxygen, it must be concluded that the direct reaction of the reductase with oxygen is without physiological importance.

3. Denaturation of the enzyme diminishes its activity with cytochrome to a much greater extent than that with oxygen, thus indicating a different mechanism for the two reactions. Denatured cytochrome reductase and the old yellow enzyme of Warburg and Christian are not identical.

4. 2,4-Dinitro-*o*-cyclohexylphenol inhibits the respiration of intact yeast cells. In isolated systems the substituted phenol inhibits the enzymatic action of cytochrome *c* reductase and of *Zwischenferment*, but it does not inhibit the enzymatic *oxidation* of cytochrome *c*. The inhibition of enzymatic reactions by this substituted phenol cannot be regarded as a specific flavoprotein inhibition.

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THE DETERMINATION OF GLUCOSE IN MINIMAL QUANTITIES OF BLOOD

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Folin and Malmros (1) have described a method for determining glucose in a tungstic acid filtrate of blood by the reduction of ferricyanide in the presence of a cyanide-carbonate buffer. The ferrocyanide formed was estimated colorimetrically after conversion to ferric ferrocyanide. Jeghers and Myers (2) modified this method, so that only 0.02 ml. of blood is necessary for an analysis. In this form the method has been serviceable in experiments on small animals in which only limited quantities of blood are readily available. It has, however, the disadvantage that not enough protein-free filtrate is produced to allow for duplicate analyses. Furthermore, when an attempt was made to use a photoelectric photometer to estimate the amount of ferric ferrocyanide, some of the ferricyanide was reduced to ferrocyanide in the blank and the amount of reduction due to a given quantity of glucose varied from one group of analyses to another.

It was found that the use of the photoelectric photometer with a properly chosen filter would allow the estimation of much smaller quantities of glucose, and that only a fourth as much filtrate as had been recommended was required for an analysis. The remainder of the filtrate could then serve as an ample supply of material for repetition of the analysis. The reduction in the amount of filtrate used also allowed a decrease in the amount of ferricyanide that was added. This in turn eliminated most of the formation of ferrocyanide which had previously occurred in the blank. In addition, the formation of ferrocyanide in the blank was found to occur only during the interval between the addition of the cyanide-carbonate buffer and the acid ferric gum ghatti solution. Since the rate of formation of the ferrocyanide ion in the blank has a negative temperature coefficient (3), a short heating period was inserted between the addition of the ferricyanide and the cyanide-carbonate buffer. This conforms with a procedure previously developed by Schott.[1] At the end of the usual heating period the tube containing the solution was cooled in ice water and the ferric gum ghatti was added as quickly as possible to reduce further the time during which the formation of ferrocyanide in the blank would be favored by a low temperature. The combination of all these procedures

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[1] Schott, H. F., personal communication to the author.

was sufficient to make the formation of ferrocyanide in the blank negligibly small.

The ferric ferrocyanide, as measured by absorption of light, was found to be formed at a progressively decreasing rate for about 30 minutes after the addition of the ferric gum ghatti solution. Alterations in temperature affected this process somewhat. Therefore, after the usual heating period, the solution was cooled to a given temperature rather than for a given time and was allowed to stand 45 minutes after the addition of the ferric gum ghatti in order to insure that the formation of ferric ferrocyanide had approximated its limit. Empirical trial further showed that if the usual heating period was increased from 8 to 15 minutes and the solution was protected from air currents during this period the reproducibility of the results was improved. The final form of the method evolved is as follows:

Reagents—

Ferric iron-gum ghatti solution. A saturated solution of gum ghatti is prepared by suspending the tears in a copper screen at the top of a tall container filled with water. About 18 hours should be allowed for saturation and 20 gm. of the gum should be used per liter of water. The solution is filtered and 7 gm. of ferric sulfate hydrate dissolved in 75 ml. of 85 per cent phosphoric acid per liter are added. Finally, small additions of 1 per cent potassium permanganate solution are made until a trace of pink persists for 15 minutes.

Cyanide-carbonate buffer. 4 gm. of sodium carbonate dissolved in 20 to 25 ml. of water are added to 0.75 gm. of sodium cyanide dissolved in 75 ml. of water. The combined solutions are then diluted to 500 ml. with water.

Potassium ferricyanide solution. 250 mg. of potassium ferricyanide are dissolved in 500 ml. of water.

Dilute tungstic acid. 10 ml. of 0.67 N sulfuric acid and 10 ml. of a 10 per cent solution of sodium tungstate are added separately to 480 ml. of water with shaking.

Procedure

0.02 ml. of blood is measured into 5 ml. of the dilute tungstic acid with a capillary pipette graduated "to contain," and mixed by a stream of bubbles blown from the tip of the pipette. After 15 minutes the mixture is centrifuged and the supernatant fluid decanted. 1 ml. of this "filtrate" is transferred to a Klett-Summerson photometer tube graduated at 5 ml. (In the studies presented here, selected $\frac{1}{2}$ × 4 inch lime glass culture tubes were substituted with good success.) 1 ml. of the potassium ferricyanide solution is then added and the tube is heated for 15 seconds in the boiling water bath. It is then removed from the bath, 1 ml. of the buffer is added, a marble is placed over its mouth, and it is replaced in the bath promptly.

The tube is now kept in the boiling water bath for exactly 15 minutes and then cooled in ice water until a thermometer in a control tube, which contains 3 ml. of water and has been heated concurrently, reads 30°. The marble should be removed during the cooling period, for when the thermometer shows 30° the tube is quickly removed from the ice water bath, and 1 ml. of the ferric iron-gum ghatti solution is immediately added. The volume is made to 5 ml. by the addition of water and the solution is thoroughly mixed. The tube is now left for 45 minutes, and the amount of
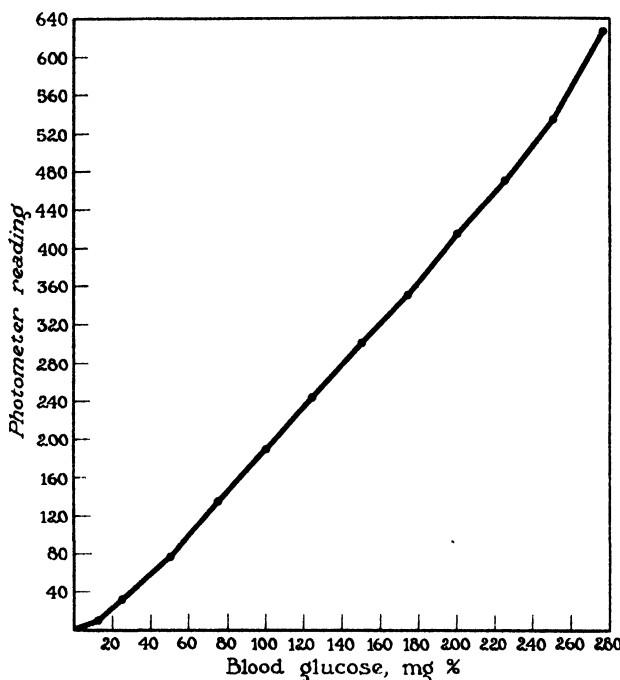
FIG. 1. Calibration curve. Culture tubes ($\frac{1}{2}$ × 4 inches) were substituted for photometer tubes; 251 mg. per cent are equivalent to 10 γ in the sample analyzed.

ferric ferrocyanide present is then estimated in a Klett-Summerson photoelectric photometer fitted with a filter which has a maximal transmission at a wave-length of about 6400 Å. (Corning filters, Nos. 243 and 978, the latter one-half standard thickness.) The photometer should be set to give a zero reading for a blank carried through the analysis with the sample and identical with it except that it contains distilled water instead of filtrate. If the sample gives a reading of more than 600 on the photometer scale the analysis should be repeated on a 0.5 ml. sample. 0.5 ml. of water must then be added to maintain the proper concentration of reagents.

The results obtained with this method have been so constant over a period of time and the amount of interfering material in the blank so minimal that it has been practical to use a calibration curve such as is shown in Fig. 1. Others (4) working with similar methods apparently

TABLE I

Analyses of Oxalated Blood to Which Known Quantities of Glucose Had Been Added and Comparison with Somogyi's Method*

The results are expressed in mg. per cent.

Glucose	Analysis by Somogyi's method	Analysis by method described			
Added....................	0	0	20	50	100
Found...	81	91	111	144	193
	82	91	111	138	191
	84	91	113	141	191
	84	87	112	137	191
	85	91	111	141	193
Average..	83.2	90.2	111.6	140.2	191.8
Glucose expected			110.2	140.2	190.2

* The unused remainder of about 100 oxalated blood samples sent to the clinical laboratory for various analyses was pooled. Five 1 ml. samples were taken from this pooled sample for analysis by Somogyi's first macroprocedure (6), and five 0.02 ml. samples for analysis by the method described. The remainder of the blood was used to fill three 50 ml. volumetric flasks containing 10, 25 and 50 mg. of United States Bureau of Standards glucose respectively. After thorough mixing five 0.02 ml. samples were taken from each for analysis by the method described.

TABLE II

Equivalency of Fructose and Glucose

Standard solutions made from United States Bureau of Standards glucose and Pfanstiehl C.P. fructose were used.

	Mg. per cent*															
Fructose present.........	13	25	50	75	100	125	151	176	201	226	251		125	25	75	100
Glucose "												125		100	50	25
Total sugar present.	13	25	50	75	100	125	151	176	201	226	251	125	125	125	125	125
" " as glucose, found............... ..	16	26	53	80	103	132	159	183	203	231	251	126	131	127	128	130

* 251 mg. per cent are equivalent to 10 γ in the sample analyzed.

have found it impracticable to use a blank to set the photometer. This, however, has been quite successful in this procedure.

If quantitative pumps (5) are used, the addition of the reagents is speeded to such an extent that as many as seventeen samples and a blank can be

carried through the procedure in a group with good results. The outlet tips of these devices should be drawn fine enough so that the reagent is ejected with enough force to insure mixing. (Tuberculin syringes fitted with some sort of a device to prevent the plunger from pulling out further than the 1 ml. mark should be used on the pumps.)

Comment

In routine laboratory practice, analyses on each sample of filtrate carried through the procedure in different groups are expected to agree within 5 per cent or within 2 units on the photometer scale. 95 per cent of a series of more than 300 analyses checked within this range on the first repetition.

Table I shows that glucose added to oxalated blood can be accounted for in a satisfactory manner by this method. It also shows that the method will give slightly higher values than Somogyi's method (6), as would be expected, since the tungstic acid filtrate used is known to contain more non-sugar reducing substances than the zinc hydroxide filtrate.

Table II shows that the method gives only very slightly higher values for fructose than for glucose and that, therefore, the method may be used in conjunction with the author's fructose method (7) to determine variations in blood glucose in fructose tolerance tests.

I wish to express my appreciation to Miss Elma Lanterman for technical assistance in chemical analyses.

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THE EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF PURIFIED YEAST CARBOXYLASE

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The components of the enzyme system in yeast, active in the decarboxylation of pyruvic acid, have been known since Lohmann and Schuster's (1) identification of cocarboxylase in 1937. Recently, Green, Herbert, and Subrahmanyan (2) have succeeded in purifying this enzyme complex and have shown it to be a diphosphothiamine-magnesium-protein. In alkaline solution this conjugated protein readily dissociates into its components.

During the course of the investigations on yeast, several workers observed that substances, other than the known components of the carboxylase system, augmented CO_2 production from pyruvic acid. Ochoa (3) and Lipschitz, Potter, and Elvehjem (4) reported that the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of Mg^{++} and either boiled yeast extracts or pure cocarboxylase was increased by thiamine. Under the same conditions, hexose diphosphate also increased CO_2 production (4, 5). Greenberg and Rinehart (6) found that cysteine, reduced glutathione, and other reducing compounds stimulated pyruvic acid decarboxylation by yeast suspensions.

Thiamine activation has been intensively studied by Lipton and Elvehjem (7). These investigators demonstrated that this effect is not the result of formation of cocarboxylase from the thiamine, as was earlier suggested. They found that the response of yeast suspensions to added thiamine differed greatly, depending on the type of yeast employed. They suggest that yeasts such as bakers', in which added thiamine causes a marked increase in pyruvic acid decarboxylation, contain material, probably protein, which can adsorb thiamine and also cocarboxylase. In the presence of excess thiamine more cocarboxylase remains unadsorbed and so is available to act as the coenzyme by combining with enzyme protein. Brewers' yeast, which is activated by thiamine to a much smaller extent, according to this view, would contain less cocarboxylase-binding material.

If this explanation of thiamine stimulation is correct, no effect would be expected if thiamine were added to the purified enzyme. Experiments with purified carboxylase are reported in this paper. It will be seen that thiamine and other substances which stimulate carboxylase activity in yeast suspensions have no effect, with the exception of cysteine, on the activity of the purified enzyme.

The method of Green, Herbert, and Subrahmanyan (2) was followed for the preparation of purified carboxylase. This method, which involves removal of impurities from the yeast extract with calcium phosphate and subsequent fractionation with ammonium sulfate, has been successfully applied to both brewers' yeast[1] and bakers' yeast. The product of the third fractionation with ammonium sulfate, designated by Green, Herbert, and Subrahmanyan as Fraction IIIb was used as the purified enzyme. 94 units (2) of carboxylase were obtained from 100 gm. of dry brewers' yeast and 73 units from 100 gm. of dry bakers' yeast. Based on dry weights from dialysis, the Q_{CO_2} of the carboxylase from brewers' yeast was 2750 and the Q_{CO_2} from bakers' yeast, 2900. Carboxylase prepared from bakers' yeast appeared to be identical with the carboxylase prepared from brewers' yeast, as no differences were observed in the action of these two purified preparations on pyruvic acid.

Carboxylase activity was determined by measuring the volume of CO_2 produced in the Warburg apparatus. The side arm of the Warburg vessels contained a solution of the substrate, 4 mg. of sodium pyruvate, and $MgCl_2$ (containing 0.1 mg. of Mg). The main vessel contained citrate buffer, pH 6.0, purified carboxylase, and the substances whose actions were to be tested. All solutions were adjusted to pH 6.0 (colorimetric) before use. The total fluid volume in the reaction vessels, after tipping, was 2.0 ml. The gas phase was air. CO_2 production was measured at 15 minute intervals for an hour. The bath containing the vessels was usually at 20°. The finding of Green, Herbert, and Subrahmanyan (2) of higher carboxylase activity in citrate than in phosphate was verified. When stored in the cold room under half saturated ammonium sulfate, the purified carboxylase retained its activity satisfactorily.

The carboxylase unit as defined by Green, Herbert, and Subrahmanyan (2) is based on the enzyme action at 30°. In the present experiments, the manometric measurements were usually made at 20°. The temperature coefficient between 20-30° was determined and found to be 1.8.

Results

Typical results of experiments with purified carboxylase are presented in Table I. In contrast to results with yeast suspensions, the rate of CO_2 production from pyruvic acid was not increased when purified carboxylase (diphosphothiamine-magnesium-protein) was supplemented with cocarboxylase[2], thiamine, histidine, or creatinine. These results demonstrate,

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[1] Obtained from the Philadelphia Brewing Company.
[2] Kindly supplied by Merck and Company, Inc.

in our opinion, that the stimulating effect of these substances involves material, other than the enzyme, present in yeast suspensions. In purified carboxylase preparations this inhibiting material is absent. Discussing the mechanism of thiamine stimulation, Lipton and Elvehjem (7) postulate that protein present in yeast, other than enzyme protein, inhibits CO_2 production by binding cocarboxylase. This conclusion is not necessarily invalidated by the finding (Table I) that the addition of inactive yeast protein (obtained by allowing alkaline washed yeast to age for a few days) to purified carboxylase does not inhibit the enzyme action. The cocarboxylase in the purified carboxylase complex is presumably all combined with enzyme protein. It at least shows that there is no reversible reaction.

In contrast to the results with thiamine and histidine it will be observed that cysteine stimulated the decarboxylation of pyruvic acid by purified

TABLE I

Effect of Various Supplements on Activity of Purified Carboxylase

Supplement	CO_2, c.mm. per 45 min.	
	Control, no supplement	With supplement
Cocarboxylase, 8 γ	142	142
" 20 "	150	145
Thiamine, 20 γ	173	170
Histidine, 5 mg.	173	174
Creatinine, 5 "	159	160
Inactive yeast, 0.1 cc.	121	122
" " 0.5 "	122	122
Cysteine, 5 mg.	172	233
" 5 "	134	183

carboxylase as well as by yeast suspensions. Cysteine appears to have a direct action on the enzyme in contrast to the indirect action of the other substances.

A large number of experiments have been done in which substances of biological occurrence were added with cocarboxylase to alkaline washed brewers' yeast and the effect of these additions on the decarboxylation of pyruvic acid observed. A stimulating effect was exhibited by histidine, histamine, and some other imidazole compounds. In spite of relatively large quantities of material used, the increase in CO_2 production was small and was of the same order of magnitude whether brewers' or bakers' yeast was used. The average increase in CO_2 was 17, 24, and 45 per cent, respectively, when 2.5, 5, and 10 mg. of histidine were added. 5 mg. of histamine produced a 38 per cent increase in CO_2 production. 5 mg. of anserine, car-

nosine, creatinine, or methylimidazole resulted in 10 to 14 per cent increases.

The stimulating effect on decarboxylation brought about by these compounds does not involve marked change in their chemical make-up, at least to an extent that modifies the diazo reaction in the case of histidine and histamine or the Jaffe reaction of creatinine. No change was found in the concentration of these substances, determined colorimetrically, during an hour's contact with alkaline washed yeast, pyruvic acid, and cocarboxylase. If cocarboxylase was omitted from the reaction, the presence of

TABLE II

Inhibition of Carboxylase by Hemin

Experiment No.	Supplement		CO_2, c.mm. per 45 min.	
			Control, no supplement	With supplement
1	0.5 mg. hemin			167
	0.1 " "		206	154
	0.5 " "			148
2	0.1 " "			121
	0.1 " "	8 γ cocarboxylase	142	122
3	• 0.25 " "			29
	0.25 " "	20 γ cocarboxylase	121	27
4	0.25 " "			22
	0.25 " "	20 γ thiamine	122	27
5	0.02 " "			122
	0.02 " "	1 mg. thiamine	149	119
6	0.02 " "			146
	0.02 " "	5 mg. histidine	161	143
7	0.25 " "			33
	0.25 " "	5 mg. histidine	115	46
8	0.02 " "			119
	0.02 " "	25 mg. creatinine	138	108

imidazole compounds did not lead to any decarboxylation of pyruvic acid. Evidently, they cannot replace cocarboxylase as a coenzyme.

When purified carboxylase was used instead of yeast suspensions, histidine had no effect on CO_2 production. This suggests that materials present in yeast but absent from purified carboxylase preparations are involved in this action.

Purified Carboxylase and Hemin—In Table II results are presented which show that small quantities of hemin inhibit the action of purified carboxylase. These experiments were suggested by the fact that histidine, histamine, and other basic substances can form hemochromogens and consideration of the possibility that the indirect effect of these compounds on

carboxylase action involved hemochromogen formation. It is of considerable interest to find in hemin a substance which inhibits the activity of purified carboxylase. Hemin-like compounds are present in yeast but absent from purified carboxylase.

The question as to whether imidazoles exert their stimulating action on CO_2 production from pyruvic acid in yeast suspensions by combining with a hemin inhibitor present in yeast cannot be definitely answered at present. As will be seen in Table II, we were unable to protect carboxylase from hemin inhibition by adding histidine or other substances that might combine with hemin. In view of these results, a mechanism of imidazole stimulation of carboxylase action in yeast which involves hemin could not be established.

SUMMARY

Purified carboxylase has been prepared from both brewers' and bakers' yeast. The temperature coefficient for carboxylase was found to be 1.8 between 20–30°.

Thiamine, cocarboxylase, histidine, and other imidazole compounds, all of which stimulate decarboxylation when suspensions of washed yeast are used, had no effect on the rate of CO_2 production from pyruvic acid by the purified carboxylase. Cysteine stimulated the action of the purified enzyme as well as the carboxylase action of yeast suspensions. Hemin was found to inhibit the activity of pure carboxylase.

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INVESTIGATIONS ON THE NATURE OF BLOOD IODINE*

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The present report concerns experiments which were undertaken to define certain characteristics of blood iodine and to shed some light on the probable nature of the circulating thyroid hormone. The authors have previously demonstrated with a permanganate acid ashing micromethod for iodine analysis that the whole blood iodine in euthyroid individuals lies within the limits of 2.4 to 4.2 γ per cent (2). The six groups of experiments to be described provide data on (1) the comparative iodine content of whole blood and serum or plasma, (2) iodine in the cerebrospinal fluid, (3) the distribution of iodine after ultrafiltration of serum, (4) the distribution of iodine during dialysis of serum, (5) the comparative iodine content of the albumin and globulin fractions of serum, and (6) the distribution of iodine in ultracentrifuged serum.

Material and Methods

All of the blood used was venous blood, drawn from human subjects with the usual strict precautions against contamination with iodine. None of the subjects had received previous iodine medication except as otherwise noted. In most cases the blood was taken in the morning before breakfast. Oxalated blood, in which the concentration of potassium oxalate did not exceed 0.2 per cent, was used for whole blood analysis and for the determination of the volume of packed red cells. Plasma was obtained by centrifugation of a portion of the oxalated whole blood. Serum was obtained from blood collected under oil in tubes and allowed to clot before being centrifuged. If a large quantity of serum was desired, blood was drawn from several individuals and the serum pooled before analysis. In the following experiments, serum and plasma have been used interchangeably, as there has seemed to be no difference in their iodine content.

The volume of packed red cells (cell volume) was determined by centrifuging oxalated blood in Wintrobe hematocrit tubes at high speed until

constant readings were obtained. Although this method is subject to considerable error (3), it was deemed sufficiently accurate because of the much larger possible error of the iodine analyses.

Serum proteins were determined by the Kjeldahl method (4). The permanganate acid ashing method of Riggs and Man (5) was used for iodine determinations. Analyses were usually made in duplicate; exceptions are noted in the tables. If the duplicates failed to check within 0.05 γ, the analysis was repeated. The aliquots taken for analysis were as large as the quantity of available material permitted; usually 10 cc. of whole blood or from 5 to 7 cc. of plasma or serum were used.

Various special procedures will be described later as each group of experiments is presented.

EXPERIMENTAL

Comparative Iodine Content of Whole Blood and Serum or Plasma—In thirteen experiments plasma or serum iodine has been compared with whole blood iodine. From these determinations and from the cell volume, the quantity of cellular iodine can be calculated. Direct analysis of packed red cells was not attempted because of the difficulty of washing the erythrocytes free from adherent serum or plasma without damaging the cells. The results of these experiments are given in Table I.

The first ten experiments were on subjects who had received no previous iodine therapy. Within the limits of experimental error, the values for iodine in 100 cc. of whole blood, and in the serum or plasma of 100 cc. of whole blood, were the same. This is further emphasized in the last column of Table I which gives the per cent of the total blood iodine accounted for by the iodine in the serum or plasma. For the six euthyroid and the two hyperthyroid subjects this varied from 90 to 111 per cent, averaging 101 per cent. Although for the myxedematous patient the values for iodine in whole blood and iodine in the plasma of 100 cc. of whole blood checked within 0.1 γ per cent, the actual values are so low that no weight can be placed on the per cent value in this instance.

It is clear from these figures that the red cells normally contain no detectable iodine. However, the last three experiments of Table I indicate that when inorganic iodine has been administered recently the serum or plasma iodine accounts for only about 70 per cent of the whole blood iodine. The remaining 30 per cent must have diffused across the red cell membrane.

Iodine Content of Cerebrospinal Fluid—Table II presents six experiments in which the iodine content of cerebrospinal fluid was determined. The spinal fluid was obtained by lumbar puncture, from patients without meningeal disease, prior to encephalography. No iodine was used in

Table I

Comparative Iodine Content of Whole Blood and Serum or Plasma

Patient	Diagnosis	Previous iodine therapy	Cell volume	Serum iodine	Whole blood iodine	Iodine in serum or plasma of 100 cc. of whole blood	Whole blood iodine in serum or plasma
			per cent	γ per cent	γ per cent	γ	per cent
R.	Normal	None	43	5.4*	3.1	3.1	100
			43	4.7*	3.0	2.7	90
C.	"	"	46	5.0*	2.7	2.7	100
E. G.	"	"	48	5.5*	3.1	2.9	94
M.	"	"	40	4.4*	2.4	2.6	108
P. G.	"	"	44	5.5	3.2	3.1	97
R. B.	"	"	46	5.0	2.5	2.7	108
W. B.	Hyperthyroid	"	47	14.8*	7.4	7.8	105
T.	"	"	41	14.0	7.5	8.3	111
Average							101
K.	Myxedema	None	38	Not > 0.8*	0.3	0.4	(133)
B.	Normal	KI 15 grains intravenously 5 hrs. previously	43	2306.0*	1928.0	1314.0	68
		KI 15 grains intravenously 49 hrs. previously	44	46.4*	35.0	26.0	74
Z.	Hyperthyroid	Lugol's solution 5 drops t.i.d.	39	27.4	24.5	16.7	68
Average of last 3 experiments..							70

* Plasma used instead of serum.

Table II

Iodine Content of Spinal Fluid

Patient	Diagnosis	Serum iodine	Spinal fluid Iodine	Spinal fluid Aliquots used
		γ per cent	γ per cent	cc.
C.	Cortical atrophy	5.0*	0.2	50
B.	Convulsions, probably Jacksonian	5.6†	0.2	50
G.	" of unknown etiology		<0.1	25
D.	Psychopathic personality	4.9	0.1	60
F.	Cerebral atrophy	8.8‡	0.4	80
L.	Cortical "	5.0‡	<0.1	35
Average		5.9	0.2	

* Plasma.
† Calculated from whole blood iodine.
‡ Single determination.

preparing the skin for the spinal tap. All the spinal fluids analyzed were normal as to protein content and cellular components. On the day of the lumbar puncture, blood was also drawn for iodine determination. While the values given in Table II for cerebrospinal fluid iodine are extremely low, they are quite reliable because of the large aliquots used for analysis. Apparently the spinal fluid contains only minute traces of iodine.

Distribution of Iodine after Ultrafiltration of Serum—The experiments on distribution of iodine between cells and serum indicated that normal circulating iodine is not diffusible across the red cell membrane, differing in this respect from administered inorganic iodine. Table III presents additional

TABLE III

Iodine in Ultrafiltrates of Serum or Plasma

Patient	Diagnosis	Ultrafiltrate		Residue		Serum or plasma iodine	
		Volume	Iodine	Volume	Iodine	Determined	Calculated*
		cc.	γ per cent	cc.	γ per cent	γ per cent	γ per cent
M.	Normal	14.9	Not > 0.3	15.2	7.7	4.4†	3.9
G.	"	21.3	0.3	14.2	11.5	5.5	4.7
B.	"	15.9	0.5	9.0	11.9	5.0	4.3
M. and R.	"	9.9	1.1	7.1	11.7	5.5	4.9
Z.	Hyperthyroid	19.3	1.0	10.5	33.3	-	11.7
M. K.	"	9.1	0.9†	8.6	18.3†		8.9
	After thyroidectomy	8.0	Not > 0.3	6.2	4.7	3.1†‡	2.1
A. K.	Myxedema	11.8	" > 0.4	9.8	1.0	Not > 0.8†	0.5
Z.	Hyperthyroid, on Lugol's solution	10.4	3.4	6.1	27.0	27.4	10.0
T.	" "	12.9	11.4	8.6	51.2	57.9	20.5

* Calculated on the assumption that all of the serum iodine is present in the residue.

† Plasma used instead of serum.

‡ Single determination.

evidence that serum iodine is not readily diffusible. Serum or plasma was subjected to ultrafiltration under mercury pressure through a cellophane membrane at room temperature for about 24 hours. By this procedure, which has been described by Lavietes (6), colloidal substances are completely retained in the substrate or residue, allowing smaller molecules to pass across the membrane into the ultrafiltrate. Iodine was measured in ultrafiltrate and residue, and when possible the whole blood iodine and serum iodine were also estimated for comparison.

In the first eight experiments, when serum or plasma from individuals who had not received previous iodine treatment was ultrafiltered, the

major portion of the iodine was retained in the residue. Indeed three of the ultrafiltrates contained no detectable iodine. As a check on this, the iodine of serum or plasma was calculated on the assumption that the ultrafiltrate contained no iodine. The calculated values agreed with the determined values within the experimental error of the method.

In the last two experiments of Table III an attempt was made to use ultrafiltration for the determination of bound iodine in the sera of hyperthyroid patients on Lugol's solution. Considerably more iodine was found in the ultrafiltrates than when no iodine therapy had been employed. Yet in both experiments the sum of the ultrafiltrate iodine and the residue iodine was far less than the iodine in the unfiltered serum. To determine whether inorganic iodine might be precipitated by contact with the mercury in the ultrafiltration apparatus, potassium iodide was added to normal serum. Part of this mixture was shaken with mercury before analysis, and part was analyzed directly. The portion shaken with mercury was found to contain only about two-thirds as much iodine as the untreated portion. Apparently some of the iodide had been rendered insoluble by contact with mercury. This finding does not invalidate the conclusion, based on the first eight ultrafiltration experiments, that virtually all of the naturally occurring circulating iodine is organically bound, for if a significant part of it had been present as inorganic iodide, the values for serum iodine calculated from the residue iodine would not have checked so well with the serum iodine values found by actual analysis.

Distribution of Iodine during Dialysis of Serum—Bound iodine was determined directly in serum freed from inorganic iodine by dialysis against 0.5 per cent iodine-free sodium sulfate solution. Control experiments indicated that added inorganic iodide was removed completely from serum by this procedure, without affecting the organic iodine originally present. A hyperthyroid subject with a serum iodine of 14.0 γ per cent and a basal metabolic rate of +41 per cent on admission was treated with 15 drops of Lugol's solution daily. After 11 days the basal metabolic rate had fallen to +14 per cent and the serum iodine was 65.7 γ per cent; yet the bound iodine (dialyzed serum iodine minus the concentration of iodine in the dialysate) was only 7.0 γ per cent. After a period of 4 days without Lugol's solution, to allow for elimination of inorganic iodine, the serum iodine was found to be 7.5 γ per cent, a value practically the same as the bound iodine when the patient was on iodine therapy. A further illustration of how dialyzed serum may be used to follow the concentration of bound iodine in the serum of patients being given Lugol's solution is presented in Fig. 1 where bound iodine and basal metabolic rate are plotted against time.

Comparative Iodine Content of Albumin and Globulin Fractions of Serum—

In three experiments (Table IV) the iodine associated with the albumin and globulin fractions of serum from hyperthyroid patients was directly determined. In the first experiment serum globulin was precipitated with 22 per cent sodium sulfate solution in the proportions of serum 1 to sodium
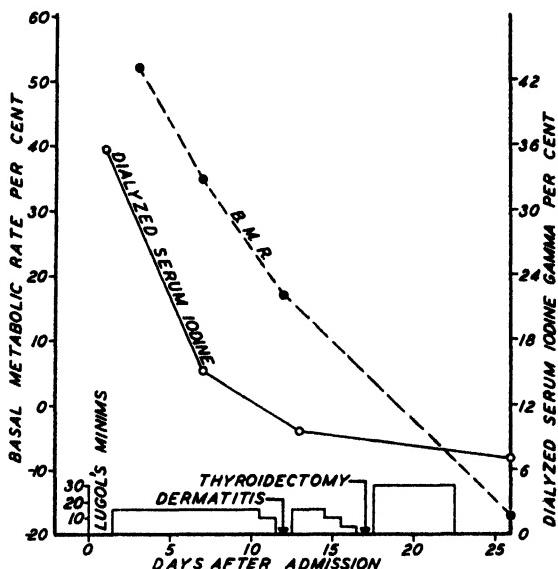
FIG. 1. Basal metabolic rate and bound iodine of a hyperthyroid patient treated with Lugol's solution before and after thyroidectomy.

TABLE IV

Distribution of Iodine between Albumin and Globulin of Serum

Patient	Pptn. of globulin			Albumin iodine			Globulin iodine	Albumin iodine + globulin iodine	Serum iodine	Albumin	Globulin	Iodine per gm. albumin	Iodine per gm. globulin
	Aliquots of serum used	Times pptd.	Recovery after last pptn.	Undialyzed portion	Dialyzed portion	Average							
	cc.		per cent	γ per 100 cc. serum	γ per 100 cc. serum	γ per 100 cc. serum	γ per 100 cc. serum	γ per 100 cc. serum	γ per cent	gm. per cent	gm. per cent	γ	γ
Pu.	6	3	49.6	14.0		14.0	4.9	18.9	17.3	4.30	2.47	3.3	2.0
Pe.	9	2	68.2	14.5	14.1	14.3	2.1	16.4	18.7	3.10	3.11	4.6	0.7
S.	10	3	62.4	15.0	16.6	15.8	3.8	19.6	18.6	4.11	3.00	3.8	1.3

sulfate 30 (4), and in the last two experiments with 24 per cent sodium sulfate solution in the proportions of serum 1 to sodium sulfate 9. The globulin was filtered on Whatman No. 50 filter paper, and the filtrate was saved for albumin and iodine analyses. The globulin was calculated as the

difference between the total protein determined in the original serum and the albumin (total protein) in the filtrate. In the second and third experiments a portion of the filtrate was dialyzed before iodine determination in order to make certain that none of the iodine in the albumin fraction was inorganic. The globulin precipitate was washed with several portions of 22 per cent sodium sulfate solution, and the washings were discarded. The precipitate was dissolved in a small quantity of 0.2 N potassium carbonate, reprecipitated, filtered, and washed again. This procedure was repeated once and the precipitate was finally dissolved in 0.2 N potassium carbonate
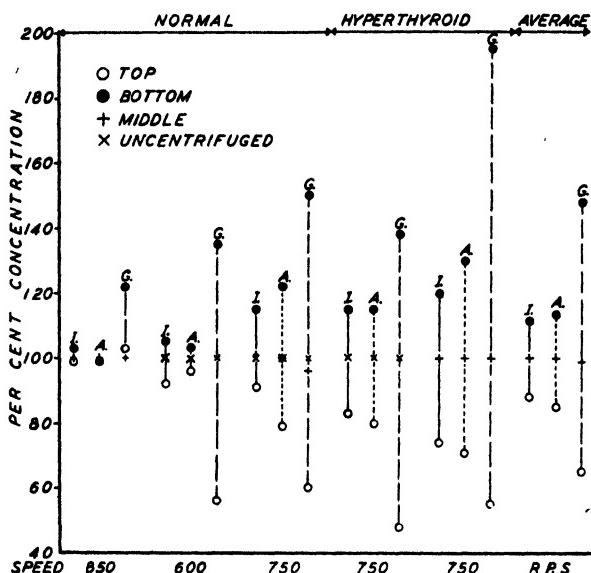
FIG. 2. Sedimentation of iodine (I), albumin (A), and globulin (G) in ultracentrifuged serum. 100 per cent on the ordinate represents the concentration in the middle or uncentrifuged portion. In each experiment, except the third, serum was centrifuged for 2.5 hours at the speed indicated. In the third experiment the serum was centrifuged for 2 hours. Centrifuge head fell off in first experiment.

and made up to a volume of 100 cc., from which aliquots were taken for protein and iodine determinations. The globulin iodine in 100 cc. of serum was calculated on the assumption that the recovery of globulin iodine after reprecipitation was proportional to the recovery of globulin.

The results indicate that, while most of the iodine was associated with the albumin fraction, a small part of the serum iodine was in the globulin fraction. However, because of the small number of determinations, the small aliquots used, and the many sources of possible error, these experiments are suggestive rather than in any way conclusive.

Distribution of Iodine in Ultracentrifuged Serum—Another approach to the problem of the nature of serum iodine has been provided by a comparison of the sedimentation rates of iodine, albumin, and globulin in ultracentrifuged serum. Pooled normal serum, or serum from untreated hyperthyroid patients, was centrifuged at speeds of from 600 to 850 revolutions per second for several hours in a Beams ultracentrifuge[1] (7). Portions of approximately equal volume were drawn off representing the top, middle, and bottom layers, or sometimes only the top half and bottom half, of the centrifuged serum. Each portion and, whenever possible, a sample of uncentrifuged serum were analyzed for iodine content. The albumin and globulin in each portion were determined by salting-out with 22 per cent sodium sulfate as described by Bruckman, D'Esopo, and Peters (4).

The results of these determinations are presented in graphic form in Fig. 2. On each vertical line the points represent the concentration of iodine, albumin, or globulin in the top, middle, and bottom portions, the concentration being expressed as the per cent of the concentration in the uncentrifuged portion or in the middle portion. Thus the length of each line is proportional to the extent of sedimentation. It is apparent that the increase in iodine concentration from top to bottom closely paralleled that of albumin, while the sedimentation of globulin proceeded much more rapidly.

<center>DISCUSSION</center>

At the present time there is no general agreement concerning the amounts of iodine to be found in the red blood cells (8–10). Our observation that the erythrocytes of both normal and hyperthyroid subjects contain little or no iodine is in agreement with that of Klassen, Bierbaum, and Curtis (11). However, the data on patients treated with inorganic iodine suggest that *inorganic* iodine is distributed fairly evenly through the water of cells and serum.

Our figures for iodine content of cerebrospinal fluid also agree with those of Klassen, Bierbaum, and Curtis (12) who found from 0.2 to 0.8 γ per cent and an average of 0.5 γ per cent of iodine in the spinal fluids of ten euthyroid and ten hyperthyroid patients. Their experimental error when they used aliquots of 15 to 25 cc. has been minimized in the present experiments by employing 25 to 80 cc. aliquots.

Most of the early work on the nature of blood iodine concerned the separation of the iodine into so called "organic" and "inorganic" fractions by the use of various organic solvents. The fallacies involved in such a separation have been adequately discussed by Salter (9, 13) and Trevor-

row (8).   The experiments on ultrafiltration and dialysis described above demonstrated that virtually all of the normally circulating iodine behaves as if it were bound to colloid particles.   These results accord with Silver's (14) observation that all the iodine usually present in blood is not dialyzable through a cellophane membrane.

Salter has suggested the separation of plasma iodine into "I" iodine, presumably iodide because ultrafilterable or non-precipitable, and into "P" iodine, "the maximum adsorbable on protein or precipitable with protein molecules" ((9) p. 75).   Salter and his associates (10, 15) have found 0.5 to 4.9 γ per cent of inorganic iodine in plasma.   The fact that in only two of Salter's twenty-four patients with varying degrees of myxedema and hyperthyroidism the inorganic iodine was as high as 4.0 γ per cent (10) renders the chance high values open to question.   Whatever the exact concentration of inorganic iodine, there is general agreement that it normally represents only a small fraction of the total circulating iodine, and that the clinically significant fraction is associated with the serum proteins.

The failure of Lerman (16) to detect thyroglobulin in the blood of euthyroid and of hyperthyroid individuals by extremely sensitive serological tests suggests that the circulating thyroid hormone is not thyroglobulin. Bassett, Coons, and Salter (10) analyzed various protein fractions in human and animal plasma and concluded that the protein-bound iodine resided chiefly in the traditional albumin fraction, as was suggested by the data in Table IV.   The similar sedimentation rates of iodine and albumin in ultracentrifuged sera also indicate that most of the iodine is associated with the albumin fraction.   As a preliminary hypothesis, the authors suggest that the circulating thyroid hormone be regarded as an iodine-containing compound of approximately the same size as serum albumin, or one of smaller size which is restrained from diffusion by serum albumin.

SUMMARY

1. In the absence of previous iodine therapy, practically no iodine can be demonstrated in erythrocytes, cerebrospinal fluid, ultrafiltrates, or dialysates of serum or plasma.

2. Dialysis of serum from patients receiving iodine therapy is a convenient method for separating inorganic iodine from the clinically significant bound iodine fraction.

3. When serum globulin is separated from serum albumin by salting-out with 22 per cent sodium sulfate solution, more of the serum iodine is found in the albumin fraction.

4. When blood serum is subjected to ultracentrifugation, the sedimentation of iodine compounds occurs at a rate very nearly equal to that of serum albumin.

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THE STRUCTURAL SPECIFICITY OF CHOLINE AND BETAINE IN TRANSMETHYLATION

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In a previous investigation (1) it was found that homocystine (or homocysteine) in the presence of choline would support growth of young rats on a methionine-free diet, whereas in the absence of choline homocystine did not replace methionine. Betaine, because of its structural relationship to choline and because of its wide-spread occurrence in natural products along with choline, was tested and found to be effective, but not so effective as choline (2).

It was noted that when rats were fed the choline-free diet, in which homocystine was the only sulfur-containing amino acid present, fatty infiltration of the livers occurred (1); the fat content of the livers was increased from the normal value of 4 to 6 per cent to 20 to 30 per cent. The possibility therefore could not be disregarded that the fatty infiltration in itself may have hindered the conversion of homocystine to methionine without a direct metabolic relationship between choline and homocystine being involved. Thus the failure of rats to grow on the homocystine diet without choline might have been due simply to damage of liver function. If this were true, it would seem likely that any compound which inhibited fatty infiltration of the liver might permit the normal process of methylation to occur. An interesting compound to try from this standpoint was triethylcholine, which has a lipotropic effect on the liver of the same order of magnitude as that of choline (3). Preliminary experiments, however, which we confirm in this paper, showed that triethylcholine did not support growth of rats on the homocystine diet, thus indicating that the ability of choline to support growth under these conditions is not dependent on the lipotropic effect. We therefore felt justified in interpreting our data as signifying a synthesis of methionine from homocystine by an actual transfer of the methyl group from choline to the reduced homocystine. The methyl groups of choline were regarded as being biologically labile (1), which enabled the choline to act as a methyl donator. Conclusive evidence in favor of this interpretation has recently been presented (4, 5).

Once the rôle of choline as a donor of methyl groups became clear, we became interested in the relationship of the structure of choline to this behavior. To elucidate the problem, the methyl-donating ability of a systematically varied group of compounds structurally related to choline

was studied. The growth of young rats on the methionine-free, homocystine-containing diet to which the compound in question was added was used as a criterion of this ability. Analogues of choline were tested, for instance, in which one or two of the N-methyl groups had been substituted by N-ethyl groups. Other analogues were used in which various substitutions had been made in the ethanol moiety. The arsenic analogue of choline was also tested, as well as various betaines and related compounds. The effect of some of the compounds on the fatty infiltration of the liver was also determined.

EXPERIMENTAL

Two kinds of diets have been used for the testing of these compounds. One was the amino acid diet devoid of methionine and cystine but containing homocystine. This diet and the vitamin supplements used have

TABLE I

Composition of HI-Hydrolyzed Casein Diet

	per cent
Hydrolyzed casein	20.0
Tryptophane..	0.4
Threonine	0.7
Histidine	0.4
Homocystine....	0.8
Crisco..............	30.0
Sucrose................	43.7
Salt mixture (Osborne and Mendel)*	4.0

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

previously been described (2). In the other diet, the amino acids were supplied by a hydriodic acid hydrolysate of casein prepared in a manner similar to that used by Welch (6). Exhaustive testing of this diet with and without choline showed the necessity of supplementation of this amino acid mixture with small amounts of histidine, threonine, and homocystine, together with the same amount of tryptophane that was used in the amino acid diet. The composition of this diet is shown in Table I. The results of the feeding experiments are shown in Table II. The amino acid diet is designated as Diet A and the hydriodic acid hydrolysate of casein as Diet B.

Several compounds other than those shown in Table II were fed. These were found to be so toxic that lethal doses consisted of only a few mg. They were tetramethylammonium chloride, neurine bromide, the betaine of β-alanine, and diethylmethylhydroxyethylammonium chloride.

Preparation of Hydrolysate of Casein—200 gm. of casein were hydrolyzed for 8 hours in 2 liters of 57 per cent (constant boiling) HI in an oil bath at

TABLE II
Compounds Tested, Growth Changes, and Food Consumption

Rat No. and sex	Days on diet	Diet	Daily supplement to basal diet	Average daily food consumption	Average daily gain or loss in weight
				gm.	*gm.*
230 ♂	24	A	No supplement	4.1	−0.1
233 ♀	24	"	" "	4.6	−0.1
265 ♂	24	"	" "	2.8	+0.1
266 ♀	24	"	" "	3.0	0.0
648 ♂	48	B	" "	3.2	−0.5
146 ♂	24	A	Choline chloride (50 mg.)	3.2	+0.9
147 ♀	24	"	" " (50 ")	3.5	+0.7
267 ♂	20	"	" " (50 ")	3.7	+1.1
268 ♀	20	"	" " (50 ")	3.6	+0.9
643 ♂	24	B	" " (50 ")	4.2	+0.8
643 ♂	12	"	No supplement	3.5	−1.8
643 ♂	12	"	Choline chloride (50 mg.)	4.1	+0.8
25 ♀	6	A	Lecithin (56 mg.)	5.0	+2.1
181 ♀	8	"	Phosphorylcholine (60 mg.)	4.9	+1.7
143 ♀	19*	"	Homocholine bromide (10 mg.)	2.0	0.0
144 ♀	12*	"	" " (10 ")	2.8	+0.3
228 ♀	24	"	" " (10 ")	3.6	−0.4
253 ♂	20	"	Arsenocholine chloride (36 mg.)	4.5	−0.4
254 ♂	20	"	" " (36 ")	4.5	−0.7
124 ♂	20	"	Triethylcholine chloride (30 mg.)	4 7	−0.7
127 ♀	20	"	Trigonelline (50 mg.)	5.1	−0.6
128 ♂	20	"	" (50 ")	4.3	−0.5
139 ♂	10*	"	Serine betaine (50 mg.)	3.2	−0.8
140 ♂	24	"	" " (50 ")	2.7	−0.1
611 ♀	14	"	Homomethionine (38 mg.)†	2.7	−0.2
612 ♂	14	"	S-Methylcysteine (46 ")†	4.0	−0.2
703 ♂	6	"	Stachydrine (40 mg.)†	5.0	−0.1
704 ♂	21	"	β-Methylcholine chloride ethyl ether (20 mg.)	1.8	0.0
249 ♂	20	"	Dimethylethylhydroxyethylammonium chloride (27.5 mg.)	5.7	+0.6
250 ♀	20	"	" "	5.9	+0.9
646 ♂	24	B	Dimethylethylhydroxyethylammonium chloride (50 mg.)	3.8	+0.6
646 ♂	12	"	No supplement	2.1	−1.1
646 ♂	8	"	Dimethylethylhydroxyethylammonium chloride (50 mg.)	3.0	+0.8
647 ♀	24	"	" "	3.9	+0.5
647 ♀	12	"	No supplement	3.3	−1.5
647 ♀	8	"	Dimethylethylhydroxyethylammonium chloride (50 mg.)	3.1	+0.6

TABLE II—Concluded

Rat No. and sex	Days on diet	Diet	Daily supplement to basal diet	Average daily food consumption	Average daily gain or loss in weight
				gm.	gm.
604 ♂	8	B	Cystine betaine (50 mg.)	4.4	0.0
605 ♂	8	"	" " (50 ")	3.6	−0.5
605 ♂	8	"	Choline chloride (50 ")	4.4	+1.2
644 ♀	16	"	Alanine betaine (50 ")	4.1	−0.6
645 ♂	20	"	" " (50 ")	4.2	−0.5
678 ♀	20	"	α,α-Dimethylcholine chloride (50 mg.)	3.6	−0.7
679 ♂	20	"	" " (50 ")	3.0	−0.5
682 ♀	20	"	Diethylmethyl-β,γ-dihydroxypropylammonium chloride (50 mg.)	2.6	−0.7
683 ♀	20	"	" "	3.5	−0.5
737 ♂	12	"	Dimethylglycine (50 mg.)	3.0	−0.9
738 ♂	16	"	" (50 ")	2.7	−0.9
739 ♂	16	"	Methyl ester dimethylglycine (50 mg.)	2.5	−1.5
740 ♂	16	"	" " " (50 ")	2.7	−0.9

* Denotes death of animal.
† Compound mixed with diet.

145–150°. Freshly distilled HI was used to which 0.5 per cent sodium hypophosphite was added as a preservative. Nitrogen was passed over the reaction mixture throughout the hydrolysis to maintain a non-oxidizing atmosphere. After 8 hours the material was assayed by the micro-Baernstein method for completeness of demethylation of methionine. If the demethylation were sufficiently complete so that less than 2 mg. of methionine per day would be present in the diet, the hydrolysis was considered satisfactory. The excess HI was then removed in vacuo. The residue was dissolved in water and the iodides were precipitated with silver sulfate. Excess silver was removed by treatment with hydrogen sulfide, and the hydrogen sulfide was removed by bubbling a stream of air through the solution. The sulfuric acid formed by the silver sulfate treatment was quantitatively removed with barium hydroxide. All precipitates were thoroughly washed by suspending them several times in hot water and the filtrate was added to the main body of material. The combined filtrates were concentrated in vacuo, were transferred to an enameled dish, and were dried in a vacuum oven at 60° over phosphorus pentoxide.

Preparation or Source of Compounds Used—Arsenocholine and phosphorylcholine were kindly furnished by Dr. A. D. Welch, serine betaine by Dr. H. E. Carter and Dr. D. B. Melville, stachydrine by Dr. H. B. Vickery, β-methylcholine ethyl ether by Merck and Company, Inc., and ergothio-

neine by Miss Eleanor Newton. We wish to express our thanks for these compounds.

Triethylcholine was prepared by the condensation of ethylene chlorohydrin with triethylamine according to the method of Channon and Smith (7), homocholine bromide by the condensation of propylene bromohydrin with trimethylamine, dimethylethylhydroxyethylammonium chloride by the condensation of ethyl iodide with dimethylaminoethanol followed by the exchange of iodide with chloride by means of silver chloride, and diethylmethylhydroxyethylammonium chloride by the condensation of methyl iodide with diethylaminoethanol followed by the exchange of iodide with chloride. Methylcysteine (8) and homomethionine (9) were synthesized by methods previously described. Cystine betaine was prepared according to Schubert (10), alanine betaine according to Novak (11), and dimethylglycine according to Michaelis and Schubert (12). The methyl ester of the latter was prepared by passing dry HCl into a solution of the dimethylglycine in absolute methanol.

α,α-Dimethylcholine chloride was prepared by the condensation of 2-amino-2-methyl-1-propanol with methyl iodide followed by the exchange of iodide with chloride. To 25 gm. of KOH in 100 cc. of water 20 cc. of the amine were added. This material was placed in a round bottom 500 cc. flask and 35 cc. of methyl iodide were added. A rubber stopper was tightly wired on the flask. The reaction mixture was allowed to stand at room temperature for 2 hours, during which time a precipitate formed. The flask was then placed in a boiling water bath for 2 hours. After separation of the inorganic salt by filtration, the filtrate was concentrated *in vacuo* at 55°. The residue was dissolved in the minimum amount of boiling 95 per cent ethanol. A portion of the residue insoluble in ethanol was found to be inorganic iodide and was discarded. Crystallization from the alcoholic solution occurred when the solution was cooled. The compound was recrystallized three times to remove inorganic iodide. The final yield of the iodide was 37 gm. The iodide content was 48.85 per cent, which agreed well with the theoretical value of 49.03 per cent.

The iodide was dissolved in water and shaken on a mechanical shaker with 37 gm. of freshly prepared silver chloride for 4 hours. After removal of the silver halides by filtration, the filtrate was concentrated *in vacuo* at 55° and the residue was dissolved in 95 per cent ethanol, from which it was precipitated as a crystalline mass by the addition of ether. This process of precipitation was repeated twice. The final yield of the chloride was 17 gm.

$C_7H_{18}ClNO$. Calculated, N 8.35, Cl 21.2; found, N 8.42, Cl 21.0

Diethylmethyl-β,γ-dihydroxypropylammonium chloride was prepared by the condensation of methyl iodide with 1-diethylamino-2,3-dihydroxypro-

pane, and by the exchange of the iodide with chloride. 5 cc. of the amine and 2.5 cc. of methyl iodide were permitted to react in a stoppered 200 cc. round bottom flask in an ice bath for 2 hours, then at room temperature for 4 hours more. A greenish yellow precipitate formed which became white when washed with ether. The material was dissolved in 95 per cent ethanol and was reprecipitated by the addition of ether. This process was repeated twice. 7 gm. of crystals were obtained. The iodide was then shaken for 2 hours with 4 gm. of freshly prepared silver chloride. After removal of the silver salts by filtration, the filtrate was concentrated *in vacuo* at 55° to a thick sirup, taken up in absolute ethanol, and precipitated by the addition of ether. It was twice reprecipitated from 95 per cent ethanol by the addition of ether.

$C_8H_{20}ClNO_2$. Calculated, N 7.09, Cl 17.9; found, N 7.12, Cl 17.7
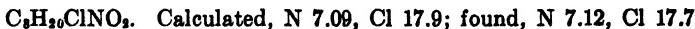
DISCUSSION

The specificity of the methylation reaction *in vivo* is well illustrated by the series of compounds employed in this study. Of all the compounds tested, in addition to choline and betaine, only dimethylethylhydroxy-ethylammonium chloride, phosphorylcholine, and lecithin promoted growth on the diets used. The activity of the phosphoric acid ester of choline and of lecithin can obviously be explained on the basis of their hydrolysis to choline in the body, although they may, of course, act directly as methyl donators. The only compound remaining which has been found to possess activity is dimethylethylhydroxyethylammonium chloride, which differs from choline in that one methyl group of the latter has been substituted by an ethyl group. If two of the N-methyl groups of choline are substituted by ethyl groups, the compound becomes toxic. Diethylmethyldihydroxy-propylammonium chloride is less toxic than the latter compound, but did not make growth possible on our experimental diet.

It will be noted that the betaines of alanine, cystine, thiolhistidine (ergo-thioneine), proline (stachydrine), nicotinic acid (trigonelline), and serine did not promote growth with homocystine in the amounts used for testing. Further work must be done with the betaine of thiolhistidine to test this compound adequately.

As shown in Table III the other known effects of choline in nutrition are not characterized by such a high degree of specificity. The comparison of the effects of the N-alkyl-substituted choline derivatives is extremely interesting. Choline itself has been shown to prevent perosis in chicks, to act as a growth essential for chicks, to enable growth of rats on a homo-cystine diet, to prevent the hemorrhagic kidneys which occur in rats on certain diets, and to prevent fatty infiltration of the liver in rats. It is of interest to note that dimethylethylhydroxyethylammonium chloride en-

TABLE III

Biological Activity of Various N- and S-Alkyl and Related Compounds

Where no reference (indicated by the figure in parentheses) is cited, evidence is presented in this communication.

Compound	Prevention of perosis	Fowl growth	Growth with homo-cystine	Prevention of kidney hemorrhage	Liver lipotropic
β-Alanine betaine			Toxic		
Aminoethanol	− (13)	− (13)	− (1)		− (14)
Arsenobetaine			−	−*	− (15)†
Arsenocholine	+ (16)	+ (17)	− (6)	+ (18)	+ (19)
Betaine	− (16)	− (16)‡	+ (1)	+ (18)	+ (14)
" aldehyde	−§	±§			+ (15)†
" " acetal					+ (15)†
Caffeine			− (1)		−‖
Calcium phosphorylcholine			+		+ (15)†
Choline	+ (16)	+ (16)	+ (1)	+ (20)	+ (21)
" methyl ether					− (22)
Creatine	− (13)	± (13)	− (23)		− (22)
Creatinine			− (1)		− (23)
Cystine betaine			−		+ (24)†
Diethylmethylhydroxyethylammonium chloride	+ (25)	− (25)	Toxic − (6)	+ (18)	+ (18)
Diethylmethyl-β,γ-dihydroxypropylammonium chloride			−		
α,α-Dimethylcholine	−§	−§	−		
Dimethylethylhydroxyethylammonium chloride	+ (17)	+ (17)	+	+ (18)	+‖
Dimethylglycine			−		
" methyl ester			−		
Dimethylthetin (sulfobetaine)				+*	+*
Ergothioneine			− (1)		− (26)
S-Ethylcysteine					+ (24)†
Glutamic betaine				−*	−*†
Homocholine			−		+ (3)
Homomethionine			−		
Lecithin	+ (27)	+ (27)	+		+ (28)
Methanol			− (1)		−‖
Methionine	− (13)	− (13)		+ (29)	+ (30)
" sulfoxide					+ (24)†
α-Methylbetaine (alanine betaine)			−		+ (15)†
β-Methylcholine	±§	−§	− (6)	+*	− (15)†
" ethyl ether			−		− (15)†
S-Methylcysteine			∓ (6)		{ − (31) + (24)†
α-Methyl-β-phenylcholine					? (15)†
Neurine bromide			Toxic		
Phosphobetaine					− (15)†
Phosphocholine				+*	+ (15)†

TABLE III—*Concluded*

Compound	Prevention of perosis	Fowl growth	Growth with homocystine	Prevention of kidney hemorrhage	Liver lipotropic
Phosphorylaminoethanol					−*†
Sarcosine			− (1)		− (23)
Serine betaine			−	−*	− (32)
Stachydrine			−		
Sulfurylcholine				−*	−*†
Tetra-β-hydroxyethylammonium chloride					− (22)
Tetramethylammonium chloride			Toxic		Toxic (33)
Threonine betaine					− (32)
Allothreonine betaine					− (32)
Triethylcholine	− (25)	− (25)	− (1)	+ (18)	+ (7)
Triethylbetaine					−*†
Trigonelline			−		−‖
Trimethylamine			− (1)		− (33)
" oxide					− (15)†
Trimethylethylammonium chloride					Toxic (33)
Trimethyl-β-hydroxypropylammonium chloride					− (34)
Trimethylphenylammonium chloride					Toxic (33)
Tripropyl-β-hydroxyethylammonium chloride				Very slight*	− (3)

* Welch, A. D., private communication.

† In these experiments the mouse was used as the experimental animal. In all other instances the rat was employed.

‡ Does not promote growth in turkeys, but has a slight growth-promoting action on chicks, amounting to less than 10 per cent of the activity of choline (Jukes, T. H., private communication).

§ Jukes, T. H., private communication.

‖ Unpublished data from this laboratory.

ables growth of rats on a homocystine diet. It also behaves similarly to choline in the prevention of perosis, promotion of chick growth, prevention of kidney hemorrhage, and prevention of fatty infiltration of the liver, but not so effectively as choline. The ability of diethylmethylhydroxyethylammonium chloride to replace choline in our diets could not adequately be ascertained because of its toxicity in the comparatively large amounts necessary. It has been found ineffective as a growth essential for chicks. It is partially effective in prevention of hemorrhagic kidney and fatty livers in rats, and apparently as effective as choline in preventing perosis in

chicks. Triethylcholine has activity only in preventing fatty liver and hemorrhagic kidneys in rats; it does not prevent perosis, or promote growth in chicks, nor does it support growth of the rat on a homocystine diet. Arsenocholine does not enable growth with homocystine, but replaces choline in its other listed functions more or less effectively.

Of particular interest is the fact that although betaine can prevent fatty infiltration of the liver and the formation of hemorrhagic kidneys in the rat and can bring about the methylation of homocysteine in this animal, it is incapable of preventing perosis or of acting to any large extent as a growth essential in the chick. Apparently the chick cannot synthesize choline directly or indirectly from betaine. In fact it would appear that the chick requires choline in the diet and cannot synthesize choline even when methionine is furnished, in contrast to the rat. On the other hand like the rat the chick can apparently transfer methyl groups from choline to bring about the synthesis of methionine from homocysteine, for homocystine plus choline can support the growth of chicks in lieu of methionine in the diet (35). Thus in contrast to the rat the transfer of methyl groups in the chick is not reversible in so far as choline and methionine are concerned.

SUMMARY

A number of derivatives of choline, various betaines, and other compounds have been tested for their ability to support growth of young rats given a diet containing homocystine but lacking in methionine. The high degree of specificity with regard to structure in relation to the ability to act as a methyl donator in this connection is shown by the fact that of the many compounds tested only choline (and simple derivatives thereof such as lecithin and phosphorylcholine), betaine, and dimethylethylhydroxyethylammonium chloride were found to support growth under these conditions.

The synthesis of α,α-dimethylcholine and of diethylmethyl-β,γ-dihydroxypropylammonium chloride has been described.

The results of the present investigation are discussed with reference to the behavior of choline and related compounds in their ability to prevent development of fatty livers and hemorrhagic kidneys in the rat and to prevent perosis and act as a growth essential in the chick.

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THE OXIDATION OF BENZOIC ACID AND RELATED SUBSTANCES BY CERTAIN MYCOBACTERIA

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The tubercle bacillus can oxidize benzoic and o-hydroxybenzoic acids.[1] It was of interest, therefore, to study the behavior of another species of *Mycobacterium*. For this purpose a non-pathogenic unnamed species from Dr. Van Niel's collection was obtained and its ability to oxidize these compounds was compared with that of the B_1 and H-37 strains of tubercle bacillus. A comparison was also made of the action of certain aromatic iodine compounds on the oxidative metabolism of these bacteria.

EXPERIMENTAL

The tubercle bacillus was grown and prepared by the method already described.[1] The non-pathogenic species was grown in flasks containing 0.2 per cent dextrose, 0.2 per cent $(NH_4)_2HPO_4$, 0.2 per cent KH_2PO_4, 0.4 per cent NaCl, 0.02 per cent $MgSO_4$, and 10 per cent tap water. For every 200 cc. of media 1.0 cc. of beef infusion broth was added. The pH was adjusted to 7.0. After 48 hours growth at 27° the bacteria were centrifuged, taken up in distilled water, and centrifuged again in modified Hopkins tubes for 15 minutes at 2000 R.P.M. The supernatant fluid was discarded and enough 0.05 M phosphate buffer, pH 6.7 or 7.8, was added so that 0.2 cc. of packed bacteria was finally suspended in 10 cc. By rotating a glass rod in the narrow part of the Hopkins tube an even suspension was readily obtained. 1.0 cc. of this suspension was used in each Warburg vessel which contained a final volume of 2.0 cc. The oxygen uptakes were measured at 37°. For purposes of comparison with the tubercle bacillus most of the experiments were carried out at pH 6.7. Despite the washing both the tubercle bacillus and the non-pathogenic species had some "resting" respiration.

Table I shows that the two strains of the tubercle bacillus and the non-pathogenic species oxidize benzoate but that o-hydroxybenzoate is oxidized only by the tubercle bacillus and m- and p-hydroxybenzoates only by the non-pathogenic species. The amino benzoates are not oxidized by any of these bacteria. The amino acids are not attacked with the exception of tyrosine which is oxidized by the non-pathogenic species only. Phenol is

[1] Bernheim, F., *J. Bact.*, **41**, 387 (1941).

readily oxidized by the non-pathogenic species but not by the tubercle bacillus. The oxidation of these ring compounds does not produce colored substances and this indicates that quinones are not formed as intermediates.

TABLE I

Oxidation of Compounds by Tubercle Bacilli and Non-Pathogenic Species

+ indicates that the compound is oxidized; − that it is not; pH 6.7, 37°.

Bacteria	Ben-zoate	o-Hydroxy-benzoate	m- and p-hydroxy-benzoates	o- and p-amino-benzoates	Tyrosine	Phenylalanine and other amino acids	Phenol	Furfural	Other aldehydes	Fatty acids	Alcohols	Succinate	Lactate, glucose, pyruvate
H-37	+	+	−	−	−	−	−	−	+	+	+	−	−
B₁	+	+	−	−	−	−	−	+	+	+	+	−	−
Non-pathogenic	+	−	+	−	+	−	+	−	+	+	+	−	+

FIG. 1. The oxidation of 0.3 mg. each of sodium benzoate, tyrosine, p-hydroxy-benzoic acid, and 0.26 mg. of phenol by a washed suspension of the non-pathogenic species at pH 6.7 and 37°. The control oxygen uptake has been subtracted. The horizontal lines represent the theoretical oxygen uptake of 8 atoms of O₂ for benzoate and tyrosine, 7 atoms of O₂ for p-hydroxybenzoic acid, and 10 atoms of O₂ for phenol.

Furfural can only be oxidized by the B₁ strain but other aldehydes, both aromatic and aliphatic, are rapidly oxidized by all three. None is able to oxidize succinate or citrate and only the non-pathogenic species oxidizes

glucose, lactate, and pyruvate. As might be expected, the parasitic tubercle bacillus is more limited in its oxidations than the saprophytic species.

Fig. 1 shows the oxidation of benzoate, p-hydroxybenzoate, tyrosine, and phenol by the non-pathogenic species at pH 6.7. There is a latent period, which is longer at pH 6.7 than at pH 7.8, before the oxidation begins. This varies from 15 minutes for m- or p-hydroxybenzoate to 60 minutes for phenol. The time for penetration into the cell probably accounts for it. The oxidation then proceeds very rapidly until the following stages in oxygen uptake and carbon dioxide production per mole are reached; benzoate, 8 atoms of O_2, 4 molecules of CO_2; m- or p-hydroxybenzoate, 7 atoms
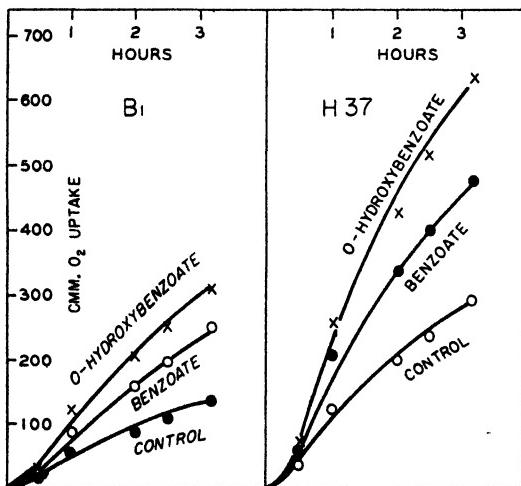
FIG. 2. The oxygen uptakes of the same amount of the B_1 and H-37 strains of tubercle bacillus alone and with benzoate and o-hydroxybenzoate. pH 6.7, 37°.

of O_2, 4 molecules of CO_2; tyrosine, 8 atoms of O_2, 3 molecules of CO_2, and 1 molecule of NH_3; phenol, 10 atoms of O_2, 4 molecules of CO_2.

When these values are attained, the oxidation rate slows abruptly but may continue to drift for some time. If a greater concentration of bacteria is used, the drift is more rapid, indicating that some substance or substances are formed which are slowly oxidized further by the bacteria. In large scale experiments, no appreciable amount of fatty or keto acids, aldehyde, or alcohol is formed, as shown by spot tests and steam distillation. This is to be expected, as these substances are readily oxidized by the bacteria. Dicarboxylic acids which are not oxidized were also shown to be absent. On evaporation to dryness only traces of organic matter are found. The conclusion is that either all four substances are eventually oxidized to CO_2

and H_2O or that the intermediates formed at the end of the rapid oxidation are incorporated into the cell.   The fact that the m- and p-hydroxybenzoate take up 1 less atom of O_2 than benzoate indicates that one or the other hydroxy derivative is the first oxidation product of benzoate.   Substitution in the ortho position completely blocks further oxidation of the molecule.   This is shown by the failure of o-hydroxybenzoate, 2,4-dihydroxybenzoate, and gallic acid to be oxidized.   The fact that tyrosine but not phenylalanine is oxidized shows the importance of the hydroxy group in this type of compound and this is again emphasized by the oxidation of phenol whereas benzene and aniline are not attacked.

The oxidation of benzoate and o-hydroxybenzoate by the two strains of tubercle bacillus is shown in Fig. 2.   No definite end-points are obtained in either case.   It is therefore not possible to say that benzoate is oxidized through o-hydroxybenzoate, although analogy with the non-pathogenic species makes it probable.   The rates of respiration of the two strains are different, and Fig. 2 shows the control or "resting" oxygen uptakes of the H-37 and B_1 strains.   The same number of bacteria were used and the uptake of the B_1 strain is less than that of the H-37.   This may be correlated with the fact that the B_1 strain grows more slowly than the H-37.   In general the control oxygen uptakes and the rapidity of the oxidation of benzoate and o-hydroxybenzoate vary somewhat with the age of the culture.   The best effects are obtained with cultures of H-37 between 10 and 14 days old and with cultures of B_1 between 16 and 21 days old.   Such cultures were used in the comparison shown in Fig. 2.   Old cultures or cultures that have turned smooth and non-virulent have very small control oxygen uptakes and lose their ability to oxidize the benzoates.   The oxidation of these substances by the tubercle bacillus differs from their oxidation by the non-pathogenic species.   With the former the rates are slower at pH 7.8 than at pH 6.7 and there is no appreciable latent period.

The question arises whether it is possible selectively to inhibit the oxidations produced by these bacteria.   For this purpose two iodine-substituted aromatic compounds were used, namely, 2,3,5-triiodobenzoate and 2,4,6-triiodophenoxy sodium acetate.   Both compounds acted in the same way.   Fig. 3 shows the effect of the second on the oxidation of various compounds by the B_1 strain.   The control oxygen uptake is inhibited 20 to 30 per cent by the drug.   The curves in Fig. 3 represent the oxygen uptakes with the substrate in the presence and absence of the drug from which the respective controls have been subtracted.   The oxidation of furfural is not significantly inhibited for 90 minutes.   After this the inhibition becomes progressively greater, presumably because at this point most of the furfural has been changed to smaller molecules which are then being oxidized by enzyme systems sensitive to the drug.   The oxidation of acetate and ben-

zoate is immediately and almost completely inhibited. On the other hand, the oxidation of o-hydroxybenzoate is not affected. Higher concentrations of the drug will, however, inhibit this oxidation also. The oxidation of benzaldehyde to benzoate is not inhibited but as soon as benzoate is formed inhibition occurs. If it is assumed that the oxidation of benzaldehyde proceeds through benzoate, o-hydroxybenzoate, lower fatty acids, and finally CO_2 and H_2O, then the drug in low concentrations selectively inhibits the second and fourth stage. Similar results are obtained with the
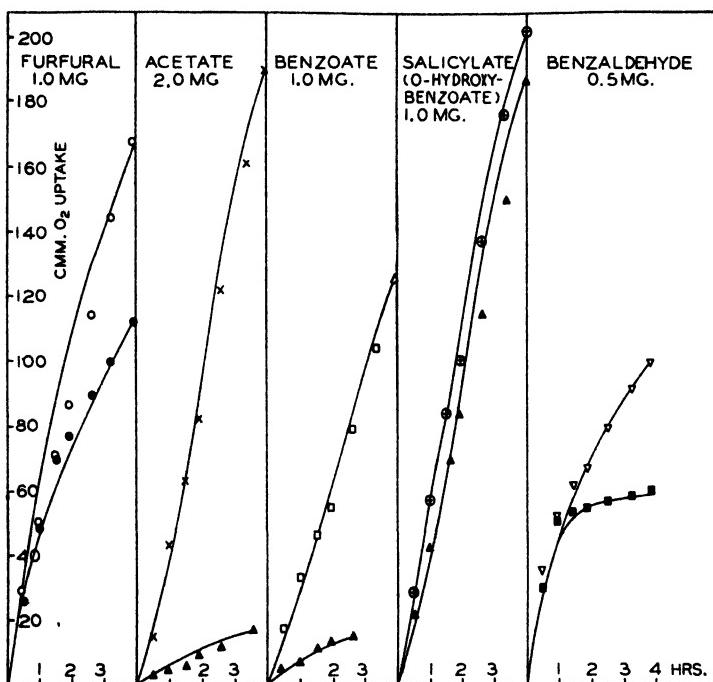
FIG. 3. The effect of 2,4,6-triiodophenoxy sodium acetate, 2×10^{-4} M, on the oxidation of various compounds by the B_1 strain of tubercle bacillus. The respective control oxygen uptakes have been subtracted. pH 6.7, 37°.

H-37 strain, with the exception, which should be emphasized, that furfural is not oxidized by this strain.

The effect of this drug on the oxidations of various substrates by the non-pathogenic species at pH 7.8 is shown in Fig. 4. The oxidation of tyrosine is most sensitive and is completely inhibited at the lowest concentration used. The oxidations of benzoate, m- or p-hydroxybenzoate, and phenol are inhibited in the sense that the latent periods are prolonged. With higher concentrations the inhibition is complete for the duration of

the experiment.   On the other hand, even the higher concentrations have
no effect on the oxidation of acetate.   This compound is very rapidly oxi-
dized by the bacteria with the uptake of 2 atoms of oxygen and the produc-
tion of 1 molecule of carbon dioxide per mole.   The bacteria readily oxidize
all the fatty acids including oleic and palmitic.
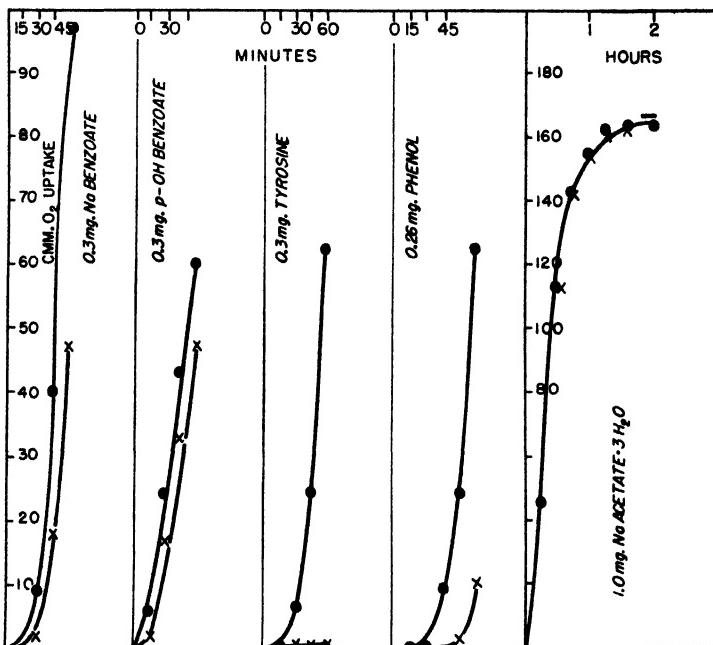
FIG. 4. The effect of 2,4,6-triiodophenoxy sodium acetate, 1×10^{-4} M, on the
oxidation of various compounds by the non-pathogenic species.   The respective
control oxygen uptakes have been subtracted.   pH 7.8, 37°.

DISCUSSION

The experiments show that the position of the hydroxy groups is impor-
tant for the oxidation of these benzoic acid derivatives by the two species
of *Mycobacteria*.[2]   This is particularly emphasized by the inability of both
species to oxidize 2,4-dihydroxybenzoate.   Substitution in the 2 position
should make it oxidizable by the tubercle bacillus, and substitution in the
4 position by the non-pathogenic species.   That no oxidation occurs indi-
cates that such substitution completely blocks the reaction.   Further spe-

[2] In the previous paper (see foot-note 1) a small oxidation of *p*-hydroxybenzoate
by the tubercle bacillus was reported.   That this was probably due to an impurity
in the preparation is indicated by the fact that recrystallization of the acid com-
pletely eliminates this small effect.

cificity has been previously shown[1] for the tubercle bacillus. Thus, if the hydroxy group of o-hydroxybenzoate is acetylated or the carboxyl group esterified, no oxidation occurs. For the non-pathogenic species methylation of the hydroxy group of tyrosine greatly prolongs the latent period but oxidation finally takes place. This was shown by comparing the oxidation of benzaldehyde and anisaldehyde. It is possible that the bacteria must demethylate the compound before oxidation can occur. It can be generally stated that amino groups inhibit the oxidation. Thus amino-substituted benzoic acids are not oxidized by either species, acetate is oxidized but not glycine, and the non-pathogenic species oxidizes lactate but not alanine, phenol but not aniline. The only deamination observed was that of tyrosine by the non-pathogenic species but, since phenylalanine is not oxidized, the liberation of ammonia must occur sometime after the breaking of the ring.

The effect of these compounds on the growth of the bacteria is being tested.

SUMMARY

1. A comparison has been made between the ability of two strains of tubercle bacillus and a non-pathogenic *Mycobacterium* to oxidize various compounds.

2. Both species oxidize benzoate. The tubercle bacillus oxidizes o-hydroxybenzoate but not m- or p-hydroxybenzoate. The non-pathogenic species oxidizes m- and p-hydroxybenzoate but not o-hydroxybenzoate. 2,4-Dihydroxybenzoate or the amino benzoates are not oxidized by either.

3. The non-pathogenic species oxidizes tyrosine but not phenylalanine, phenol but not aniline. The tubercle bacillus does not oxidize any of these compounds.

4. Both species oxidize all aromatic and aliphatic aldehydes tried with the exception of furfural. This compound is oxidized only by the B_1 strain of the tubercle bacillus. Both species oxidize fatty acids and alcohols.

5. Amino acids other than tyrosine, succinate, and citrate are not oxidized by either species. The non-pathogenic species but not the tubercle bacillus oxidizes glucose, lactate, and pyruvate.

6. The effect of two iodine-substituted aromatic compounds has been studied on the oxidations of these compounds by the two species.

NOTE ON THE SARCOSINE OXIDASE

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In a previous paper (1) an enzyme was described which catalyzes the oxidative demethylation of sarcosine to glycine and formaldehyde. The distribution, pH optimum, and specificity of the enzyme were studied. The following work was undertaken to define further the properties of the enzyme by means of its reaction with certain drugs.

EXPERIMENTAL

The enzyme preparation was obtained from rat liver by the method already described (1). 1.0 cc. of this preparation was used in each Warburg vessel which contained a final volume of 2.0 cc. 2.0 mg. of sarcosine, an amount sufficient to saturate the enzyme, was added and all the experiments were carried out at pH 7.8. The effect of several concentrations of NaCN on the oxidation of sarcosine was compared to the effect on the oxidation of succinate by the same liver preparation, since succinate is known to be oxidized through the cytochrome-cytochrome oxidase system. The results are shown in Fig. 1. As no carbon dioxide is liberated in either reaction and as the residual respiration of the enzyme preparation is very small, NaOH was not used in the inset in this experiment. The succinate curves show the gradual release from the cyanide inhibition with time due to the progressive destruction of the cyanide. With NaOH in the inset similar curves are obtained but more rapidly because cyanide is lost by distillation. In contrast to the succinate, the sarcosine curves show no recovery as the cyanide concentration decreases. The respective percentage inhibitions by the three concentrations of cyanide, 0.5, 1.0, and 2.0×10^{-3} M, on the oxidation of succinate and sarcosine are as follows: after 10 minutes, succinate, 85, 95, 97 per cent; sarcosine, 44, 66, 72 per cent; after 20 minutes, succinate, 72, 88, 93 per cent; sarcosine, 31, 50, 72 per cent. These differences in percentage inhibition can be explained by the fact that the oxidation of sarcosine is much slower than that of succinate, so that the fraction of cytochrome oxidase uninhibited by cyanide is less limiting for the former than for the latter. This is substantiated by similar experiments on the choline oxidase which also utilizes the cytochrome oxidase system and is oxidized at a rate intermediate between those of sarcosine and succinate. Under the same conditions the percentage inhibition at 10 minutes

in the presence of 0.5×10^{-3} M cyanide is 65. Thus, the cyanide effect on the oxidation of sarcosine can be fully explained by the inhibition of the cytochrome oxidase, and the fact that hydrogen peroxide is not produced during the oxidation, proved by the absence of methemoglobin formation from added hemoglobin (2), and the fact that cyanide has no effect on the methylene blue reduction by sarcosine, confirm this. The lack of recovery of the sarcosine oxidase from the cyanide inhibition is due to the instability of the enzyme. When a larger amount of cytochrome oxidase
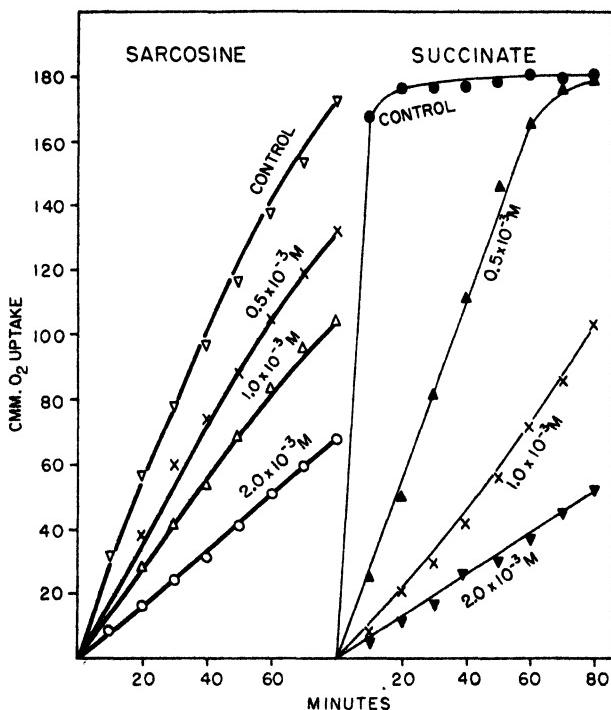
Fig. 1. The effect of various concentrations of NaCN on the oxidation of sarcosine and succinate by the same liver preparation. pH 7.8, 37°.

becomes available as the cyanide concentration decreases with time, progressive destruction of the enzyme has taken place as is shown in the following experiments.

Table I shows the effect of incubation with and without trypsin on the sarcosine oxidase compared to the effect on succinoxidase and cytochrome oxidase present in the same preparation. It is obvious that the sarcosine oxidase loses its activity much more rapidly than either of the other two enzymes. This inactivation of the sarcosine oxidase is therefore not due

to destruction of cytochrome oxidase or the destruction of the catalyst intermediate between the succinodehydrogenase and the cytochrome oxidase which may be a flavoprotein (3) or cytochrome b (4). It follows that the protease present in the preparation and the added trypsin must hydrolyze the sarcosine dehydrogenase or an intermediate between it and the cytochrome system. To determine which is the case, the methylene blue reduction by the sarcosine oxidase was tested after the oxygen uptake had completely disappeared. At this time methylene blue was still rapidly reduced by the oxidase in the presence of sarcosine. From these experiments it is possible to state that the sarcosine oxidase, like the succinoxidase, consists of at least three parts, a dehydrogenase, the cytochrome system, and an intermediate. The evidence indicates that the intermediate of the sarcosine oxidase differs from that of the succinoxidase.

TABLE I

Effect of Incubation with and without 2.0 Mg. per Cc. of Pancreatin (Merck) on Activity of Sarcosine Oxidase, Succinoxidase, and Cytochrome Oxidase

p-Phenylenediamine was used as the substrate of the last. The preparation was incubated without shaking at 37°. At the given intervals aliquots were placed in the Warburg vessels and the rate of oxygen uptake measured every 10 minutes for 40 minutes. The figures below are expressed in terms of per cent of the initial rates which in each case are given the value of 100.

Substrate	Oxygen uptake					
	Incubated alone			Incubated with trypsin		
	45 min.	100 min.	140 min.	45 min.	80 min.	105 min.
Sarcosine	100	42	35	36	22	14
Succinate	100	80	69	100	19	6
p-Phenylenediamine	100	100	100	100	76	55

The dehydrogenase part of the succinoxidase contains sulfhydryl groups that are essential to its activity (5). This can be demonstrated by incubating the enzyme preparation with small amounts of copper sulfate and testing the activity of the enzyme at intervals. If 5×10^{-4} M copper sulfate is added to the liver preparation and the activity tested immediately, there is no inhibition of the succinoxidase but a 45 per cent inhibition of the sarcosine oxidase. After 50 minutes shaking with the copper at 37° the succinoxidase is inhibited 79 per cent and the sarcosine oxidase, despite a loss of activity in the control due to the incubation, is still only 46 per cent inhibited by the copper. This experiment indicates that sulfhydryl groups are not essential for the activity of the sarcosine oxidase and that the metal acts by combining immediately with some active group. That the enzyme contains no oxidizable groups is indicated by the fact that the

"spontaneous" inactivation occurs at the same rate aerobically and anaerobically.

DISCUSSION

The sarcosine oxidase with the succinoxidase, choline oxidase, l-proline oxidase, and others is associated with the insoluble protein fraction of liver. Attempts to get this fraction into solution as a first step to separating and purifying the enzymes have thus far failed. Because the succinoxidase is present in other tissues from which it can be obtained in a purified form, its properties can be used for comparison. Thus by a study of the effects of various agents on the succinoxidase in the liver fraction it is possible to obtain information about the other enzymes present in it.

SUMMARY

1. The oxidation of sarcosine by the sarcosine oxidase goes through the cytochrome-cytochrome oxidase system.

2. The evidence indicates that an intermediate catalyst exists between the dehydrogenase and the cytochrome and that this is different from the one in the succinoxidase system.

3. The activity of the sarcosine oxidase is not dependent on sulfhydryl groups.

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AN ALIGNMENT CHART FOR THE COMPUTATION OF ULTRA-CENTRIFUGATION RESULTS

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The discovery made by Svedberg (1) that the ultracentrifuge could be adapted to the study of proteins has resulted in wide-spread use of this instrument. A convenient way of describing sedimentation data is in terms of the sedimentation constant, S, originated by Svedberg. This quantity is simply the sedimentation velocity the material would have in a unit centrifugal field.

$$S = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \tag{1}$$

Since the sedimentation rate of a given material is a function of the density of the solution and the solute and of the viscosity of the solvent, as well as of the magnitude of the centrifugal field, it is necessary to express sedimentation constants in terms of a reference solvent, such as water at 20°.

$$S_{20}^0 = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \cdot \frac{\eta_t}{\eta_{20}^0} \cdot \frac{1 - V_{20} \rho_{20}^0}{1 - V_t \rho_t} \tag{2}$$

The meanings of the various terms in these two equations are defined by Svedberg (1). When Equation 2 is integrated, S_{20}^0 is expressed as a function of $\log_e x$, t, and the other variables listed above. It is obvious that considerable arithmetic is involved in the evaluation of sedimentation constants in this manner.

It has been the experience of the author that laborious routine computations of this sort can often be reduced to very simple operations by using some sort of an alignment chart (2). If one constructs three parallel equidistant lines perpendicular to and intersecting a base-line, then, by simple geometry, any line connecting points on the outside lines will intersect the center line at a point whose distance above the base-line is one-half the sum of the distances of the other two points above the base-line. If equal scales are laid out on the two outside lines, A and B, and a scale of half the magnitude is laid out on the inside line, C, then the numerical value

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of C is equal to the sum of the numerical values of A and B (3). A device of this sort, commonly referred to as a nomograph or an alignment chart, is unusually well adapted to performing mathematical operations such as evaluating u when $u = x + y - z$. Since the sum of $x + y$ is the only quantity represented on the center scale and since its value is not recorded in a case such as this, the scale on the center line can be omitted. Similarly, an alignment chart can be constructed to solve the equation $Au = Bx^lCy^n/Dz^m$, where A, B, C, D, l, m, and n are constants. In this example, the values of each variable would be plotted on a logarithmic scale, expanded by the value of its particular exponent, and displaced vertically by the value of the logarithm of its coefficient.

Equation 1 may be integrated approximately as follows (1): $S = 1/\omega^2\bar{x} \cdot \Delta x/\Delta t$, where Δx and Δt are small but finite increments of x and t, respectively, and $\bar{x}$ is the average value of x in the interval between x and $x + \Delta x$. It can be shown that, if the experimental conditions are so chosen that Δx does not exceed $\bar{x}/10$, the error attending this approximation is less than 0.1 per cent. By inverting and by converting angular velocity to R.P.M., velocity to mm. per minute, and S to units 10^{13} times the fundamental unit,[1] this equation becomes

$$\frac{1}{S} = \frac{\pi^2}{1.5 \times 10^{13}} \cdot \frac{n^2 \bar{x}}{\Delta x/\Delta t}$$

where n represents R.P.M. This equation can be written in the form,

$$\frac{A}{S} = \frac{Bn^2 C\bar{x}}{D(\Delta x/\Delta t)}$$

where A, B, C, and D are arbitrarily assigned constants obeying the sole restriction, $BC/AD = \pi^2/(1.5 \times 10^{13})$.

$$\therefore \quad [\log A - \log S] = [\log B + 2 \log n] + [\log C + \log \bar{x}] - \left[\log D + \log \frac{\Delta x}{\Delta t}\right]$$

Since Equation 1 could be expressed in the above form, an alignment chart for its solution could be constructed, as is shown in the right half of Fig. 1. On Column I, $[\log D + \log \Delta x/\Delta t]$ is plotted as a function of the variable, $\Delta x/\Delta t$, and $[\log B + 2 \log n]$ as a function of n or R.P.M. On Column III, $[\log A - \log S]$ is plotted as a function of S and $[\log C + \log \bar{x}]$ as a function of $\bar{x}$. Convenient values, subject to the restriction stated above, were chosen for the constants A, B, C, and D.

[1] By unanimous vote at the conference on "The ultracentrifuge" held by the New York Academy of Sciences on November 14–15, 1941, it was agreed that it be proposed that this unit, 10^{13} times the fundamental unit, be called the Svedberg. The alignment chart here described gives the results in Svedbergs.

ALIGNMENT CHART FOR EVALUATING
SEDIMENTATION CONSTANTS

$a) o \iota M PO_4^{=} - pH \gamma$
$b) o \imath M NaCl$
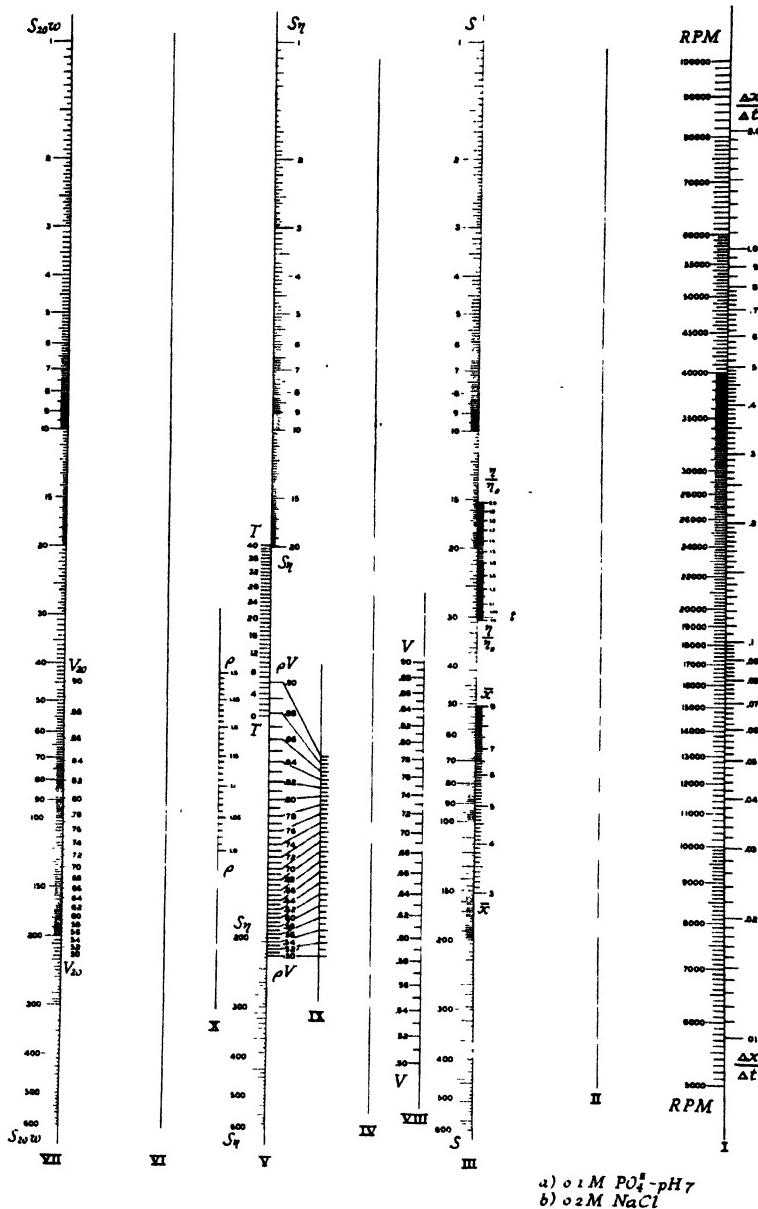
Fig. 1

With this portion of the alignment chart, sedimentation constants can be computed from ultracentrifuge data. If the sedimentation constants are to be useful for further interpretation, however, they must be corrected for the viscosity and the density of the solvent, as shown by Equation 2. This correction can be carried out in two steps,

$$S_\eta = S \cdot \frac{\eta_t}{\eta_{20}^0} \quad (3) \qquad \text{and} \qquad S_{20}^0 = S_\eta \cdot \frac{1 - V_{20}\rho_{20}^0}{1 - V_t\,\rho_t} \quad (4)$$

where S_η is the sedimentation constant corrected only for viscosity. One may assume that $\eta_t = [\eta/\eta_0]_{T_i} \times \eta_0$, where $[\eta/\eta_0]_{T_i}$ is the ratio of the viscosity of the solvent to that of water at some convenient temperature, T_i, and η_0 is the viscosity of water at the temperature of the solvent at the time of the experiment. Hence, Equation 3 may be written,

$$S_\eta = S[\eta/\eta_0]_{T_i} \times (\eta_0 = f(T))/(\eta_{20}^0 = 0.010087)$$

$$\therefore \quad [\log E - \log S_\eta] = [\log A - \log S]$$

$$+ [\log F - \log \eta_0 = f(T)] - [\log G + \log [\eta/\eta_0]_{T_i}]$$

where A, E, F, and G are arbitrarily assigned constants obeying the sole condition that $AF/EG = \eta_{20}^0 = 0.010087$. In this form an alignment chart was constructed for the solution of Equation 3 as shown on Columns III and V. Here the bracketed quantities in the above equation are plotted as functions of the respective variables S_η, S, T, and $[\eta/\eta_0]_{T_i}$ (η/η_0 in the figure). The relationship between η_0 and T that was used was that given by Svedberg and Pedersen (1).

Equation 4 yet remains to be solved. It can be written in the form $[\log H - \log S_{20}^0] = [\log E - \log S_\eta] + [\log J - \log (1 - V_{20}\rho_{20}^0)] - [\log K - \log (1 - V_t\rho_t)]$, where $EJ/HK = 1$. Columns V and VII of Fig. 1 are the alignment chart for the solution of this equation. The bracketed quantities in the above equation are plotted as functions of the respective variables, S_{20}^0, S_η, V_{20}, and $V_t\rho_t$.

Since $\log V_t\rho_t = \log V_t + \log \rho_t$ and $[\log L + \log V_t\rho_t] = [\log M + \log V_t] + [\log N + \log \rho_t]$, where $MN/L = 1$, $V_t\rho_t$ can be evaluated from V_t and ρ_t by constructing three equidistant parallel lines, with equal logarithmic scales representing V_t and ρ_t, respectively, on the extremities, and a logarithmic scale of half the dimensions representing $V_t\rho_t$ on the central column. Columns VIII, IX, and X represent the alignment chart for the valuation of $V_t\rho_t$. The lines between Columns IX and V connect values of $V_t\rho_t$ on Column IX with the corresponding values of $\log K - \log (1 - V_t\rho_t)$ on Column V.

To facilitate the use of this alignment chart, a photostatic copy was made and was mounted on a bakelite board.[2] Narrow slits were cut

[2] Photostatic copies of Fig. 1, 12 × 18 inches in size, can be obtained from the author.

through the photostat and into the bakelite in the positions of the three vertical lines, Columns II, IV, and VI, of Fig. 1. A brass key was cut to fit the channels and slide through them freely. A long thin strip of celluloid with a black hair-line through its center parallel to its two long sides was then fastened at its exact center to the brass key by means of a pivot. This device makes it possible to use the alignment chart without marking it in any way. Fig. 2 is a photograph of the mounted alignment chart showing the hair-line assembly.

The use of the alignment chart for the calculation of sedimentation constants may be illustrated with the following example. A sedimenta-
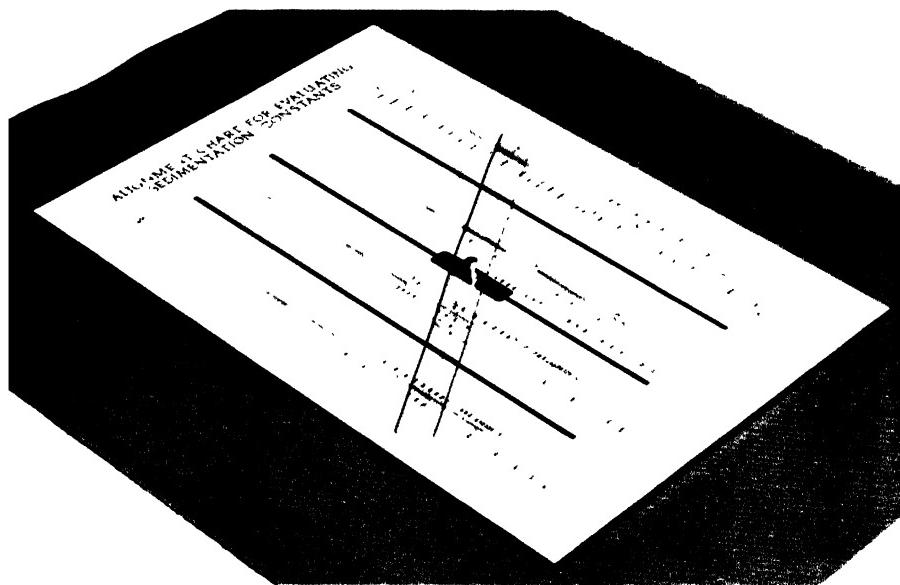
FIG. 2. Photograph showing the alignment chart mounted on a bakelite panel and equipped with convenient hair-line assembly.

tion experiment was carried out with tomato bushy stunt virus dissolved in 0.2 M NaCl and 0.01 M phosphate buffer at pH 7. The temperature throughout the run was 22.8° ± 0.2°. The centrifuge was operated at a constant speed of 18,500 R.P.M. The density of the solution was 1.01, the relative viscosity of the solvent, $[\eta/\eta_0]_{T_t}$, was 1.02, and the partial specific volume of the virus at 20.0° and at 22.8° was about 0.739. The boundary was photographed at 10 minute intervals. Other pertinent data are presented in Table I.

From the data recorded in the first two columns of Table I, $\bar{x}$ and $\Delta x/\Delta t$ can readily be evaluated. For each value of $\bar{x}$ and $\Delta x/\Delta t$, S was evaluated

by means of the alignment chart. This was accomplished by centering the hair-line assembly on Column II, by moving it into position so that 18,500 on the R.P.M. scale in Column I can be lined up with the particular value of $\bar{x}$, say 5.762 cm., on Column III. Then the hair-line was rotated about its pivot until the line coincided with the proper value of $\Delta x/\Delta t$ on Column I, in this case 0.175 mm. per minute. When this was done, the hair-line also crossed the sought value of S on Column III, 135 in this example. This process was repeated for all of the pairs of $\bar{x}$ and $\Delta x/\Delta t$ recorded in Table I, and the resulting values of S were averaged. In the last two columns of Table I, the values of S determined with the chart and those calculated arithmetically are compared. The agreement is seen to

TABLE I

Typical Sedimentation Data

t	x	$\bar{x}$	$\dfrac{\Delta x}{\Delta t}$	S, chart	S, calculated
min.	cm.	cm.	mm. per min.		
0	5.675				
		5 762	0.175	135	135
10	5.850				
		5 943	0.187	140	140
20	6.037				
		6.134	0 195	142	141
30	6.232				
		6.328	0 193	135	135.5
40	6.425				
		6.522	0 194	131 5	132
50	6 619				
Average				136.7	136.7

be excellent. The average value of S was next corrected for viscosity. This was done by centering the hair-line assembly on Column IV. The average value of S, 136.7, on Column III was aligned with 22.8 on the T scale on Column V, the hair-line was rotated and connected with 1.02 on the $[\eta/\eta_0]_{T_t}$ (η/η_0 on the figure) scale on Column III. The line was found to intersect Column V midway between 0.70 and 0.71 on the $V_t\rho_t$ scale. This point was recorded. If the S_η scale were continuous in this region, we could have read off the value of S_η directly. However, since the numerical value of this function is of no use, it is satisfactory to let it be represented by the point midway between 0.70 and 0.71 on the V_t scale. Next, it was necessary to evaluate $V_t\rho_t$. This was done by centering the

hair-line assembly in either Column IV or VI, and then by aligning the value 1.01 on the ρ_t scale on Column X with 0.739 on the V_t scale on Column VIII. The value of $V_t\rho_t$ was found at the point of intersection of the hair-line with the V_t scale on Column IX. This value, in this case 0.746, was also recorded. In order to carry out the final evaluation of S_{20}^0, the hair-line assembly was then centered on Column VI, and the value of S_η, represented by the point midway between 0.70 and 0.71 on the $V_t\rho_t$ scale of Column V, was aligned with the value of V_{20} on Column VII, 0.739. The hair-line was rotated and connected with the value of $V_t\rho_t$ on Column V, 0.746, and the value of S_{20}^0 was then read off at the intersection of the hair-line with Column VII. The value in this case was 134. When S was corrected arithmetically, the value of S_{20}^0 was found to be 134. In another sample computation in which values near the ends of the scales were used, the value computed by means of the alignment chart was found to agree within about 1 per cent with the true value computed arithmetically.

As was demonstrated in the examples just discussed, with the aid of the alignment chart it was possible to compute the corrected sedimentation constant of bushy stunt virus without referring to anything except the original data. All of the auxiliary information needed is incorporated into the device itself. It was even possible to perform some necessary operations which cannot be carried out with other devices such as the standard slide-rule—for example, the evaluation of the expression $(1 - V\rho)$ from V and ρ. When these and other factors are taken into consideration, it is obvious that the calculation of sedimentation constants from experimental data by means of the alignment chart is a much simpler and faster process, on the whole, less subject to mistakes, than any of the more direct arithmetic processes, no matter how well systematized. The actual time required to perform the operations described above is about 3 minutes. If the computation is well organized and various tables of data are before the operator, the same operations can be carried out in about 10 minutes with the slide-rule. If the exact integral of Equation 1 is used, it is necessary to convert distances into logarithms and the time of computation is thereby considerably increased. On the whole, therefore, the alignment chart is capable of saving considerable time, and this, as has been demonstrated, can be realized without any appreciable sacrifice of precision.

SUMMARY

An alignment chart for the evaluation of sedimentation constants from sedimentation data was constructed and its use in a sample computation was described. It was demonstrated that this device is accurate, convenient, and capable of effecting a considerable economy in time.

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ORGANIC PHOSPHATE AND "FRUCTOSE" IN RAT INTESTINAL MUCOSA, AS AFFECTED BY GLUCOSE AND BY PHLORHIZIN

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In contrast to absorption of the hexoses, sorbose and mannose, and of the pentoses, xylose and arabinose, absorption of the physiologically important hexoses, galactose, glucose, and fructose, by the mucosa of the small intestine is a comparatively rapid process, and does not proceed in accordance with the laws of diffusion (1, 33). Absorption of these sugars is therefore spoken of as active, in contrast to the passive absorption of the physiologically unimportant sugars.

Verzár and his coworkers have postulated that the rapid absorption of glucose, etc., is due to phosphorylation of these hexoses, *i.e.* to a coupling of the hexose molecules with phosphate, with a consequent setting up of a much steeper diffusion gradient for these sugars, and that the passively absorbed sugars are not appreciably phosphorylated. Part of the evidence which they have advanced in favor of this hypothesis is (1) that iodoacetic acid, which interferes with enzymatic phosphorylation processes (16), also markedly inhibits absorption of the three hexoses, but not that of xylose and arabinose (33); (2) that the acid-soluble organic phosphate content of rat intestinal mucosa is considerably increased during absorption of the three hexoses, but not during the slow passive absorption of xylose, arabinose, and mannose (7, 9); and (3) that iodoacetic acid prevents the increase in acid-soluble organic phosphate otherwise secured on absorption of the three hexoses (29).

Klinghoffer (8) and Öhnell and Höber (21) have criticized conclusions drawn from the use of iodoacetic acid, on the grounds that this agent is very toxic, that it produces a bloody and irreversibly damaged intestinal mucosa, and that in their experiments it interfered with the absorption of other substances, *i.e.* NaCl and xylose, as well as with that of the hexoses.

These objections cannot be raised to anything like the same extent against the use of phlorhizin in similar studies. Since the first experiments by von Mering (18) it has been known that the intense glycosuria and polyuria produced by this agent are reversible, even after prolonged administration. Walker and Hudson (31) have demonstrated by direct observation that in the amphibian kidney phlorhizin acts to prevent reabsorption

of glucose by the proximal tubule cells.   The work of Nakasawa (20) and of others (17, 21, 32) has demonstrated that this agent is also capable of markedly and yet reversibly inhibiting the absorption of the physiologically important hexoses by the small intestine.

As regards specificity, Lundsgaard (17) has reported that 0.02 M phlorhizin does not affect intestinal absorption of glycine or of glutamic acid (rabbit and cat) and Öhnell and Höber (21) have reported failure of this agent to inhibit absorption of the amino acid asparagine (rat).   In both cases a simultaneously occurring absorption of glucose was markedly inhibited by the phlorhizin.   Verzár and Laszt (28) have reported inhibition of fat absorption in rat intestine by phlorhizin, and considered this effect due to inhibition of fatty acid phosphorylation.   Considerably higher concentrations of phlorhizin were required than were required to interfere markedly with glucose absorption.

Since phlorhizin is relatively non-toxic, and its effects are reversible and specific, experiments have been performed similar to those of Verzár and his coworkers, but with iodoacetic acid replaced by phlorhizin.

<div align="center">EXPERIMENTAL</div>

Phosphate Fractions in Trichloroacetic Acid and Derived Extracts of Rat Intestinal Mucosa

Phosphate Determinations—Inorganic orthophosphate was determined by the method of Fiske and Subbarow (3).   Total phosphate was determined by wet ashing, to convert all the organic phosphate to inorganic orthophosphate, as described by Fiske and Subbarow, but with nitric acid replaced by superoxol as the oxidizing agent.   The color determinations were made with a Coleman spectrophotometer set at 500 mμ.

Organic phosphate hydrolyzed within 7 minutes by N HCl at 100° is called 0 to 7 minute organic P or pyrophosphate P (see (12)).   Additional organic phosphate hydrolyzed by further heating to 180 minutes with N HCl at 100° is called 7 to 180 minute organic P.   Organic phosphate not hydrolyzed within 180 minutes by the N HCl is called difficultly hydrolyzable organic P (see (14)) and equals total phosphate minus inorganic phosphate present after the extract is heated with the N HCl at 100° for 180 minutes.   Some of the organic phosphate compounds which correspond to these various fractions are shown in Table I.

In the experiments in which no separation by barium precipitation was carried out any HCl used in hydrolysis was neutralized with NaOH, and molybdate solution containing 0.3 N H_2SO_4 was used to develop color, as described by Fiske and Subbarow.   In the barium separation experiments the HCl[1] used for hydrolysis was also used for color development.   Addi-

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[1] Special spectrophotometer tests at 500 mμ with standard inorganic phosphate solutions gave the following results.   (1) During the first few minutes the blue color

tional HCl was added to bring its concentration to a final value of 0.5 N in determinations on barium filtrate and barium precipitate solutions, and to a final value of 0.3 N in determinations on trichloroacetic acid filtrates. Even with H_2SO_4 replaced by HCl some precipitate was secured in the barium filtrate determinations, on addition of the aminonaphtholsulfonic

TABLE I

Scheme for Estimation of Changes in Some Organic Phosphate Compounds Present or Expected in Intestinal Mucosa

		Total phosphate (determined by converting all organic phosphate soluble in trichloroacetic acid into inorganic phosphate)		
	Direct phosphate	Total organic phosphate (total PO₄ minus direct PO₄)		
		Organic phosphate hydrolyzed by N HCl at 100°		Organic P not hydrolyzed to inorganic P by N HCl even after 180 min.
		Between 0–7 min.	Between 7–180 min.	
Compounds having relatively soluble barium salts	Creatine phosphate (10)	Glucose-1-PO₄ (2) (only small amounts, if any, present)	¼ Robison, ¾ Neuberg ester (10, 13) Triose PO₄ (19) Appreciable amounts adenylic acid (22)	¾ Robison, ¼ Neuberg ester (10, 13) Greater part adenylic acid (22)
Compounds having relatively insoluble barium salts	Inorganic phosphate (10, 22)	Adenosine di- and triphosphate (11, 12, 22) About 25–30% hexose diphosphate (10)	About 70% hexose diphosphate (10)	Phosphoglyceric acid (15) (only small amounts, if any, present) About 5% hexose diphosphate (10)
		Accumulation of pyrophosphate P not affected by phlorhizin	Newly formed carbohydrate-PO₄ should be accumulated in these two fractions. Inhibitory effects of phlorhizin exhibited here	

The figures in parentheses represent bibliographic reference numbers.

produced by mixing molybdate and inorganic phosphate appears more rapidly in the presence of 0.5 N sulfuric acid (final concentration) than in the presence of 0.5 N HCl. (2) After 60 minutes the color development is practically complete, when either acid is used, and is somewhat stronger if HCl is used. (3) In both cases a straight line is obtained if log per cent transmission is plotted against concentration of inorganic phosphate (over the range of 0.002 to 0.06 mg. of P as inorganic orthophosphate in a final volume of 10.0 cc.). (4) If the concentration of HCl is halved, an intense blue color develops, and the amount of color bears no relation to the amount of inorganic phosphate employed; if the concentration of HCl is doubled, the color development becomes very slow.

acid reagent, but this was fairly readily centrifuged down, and did not appear to interfere with the determinations. Added inorganic phosphate was completely recovered.

Preparation of Trichloroacetic Acid Filtrates—The rats were fasted for 24 (occasionally 48) hours, then given the test solution, warmed to about 37°, by stomach tube. 1 cc. of test solution was given for each 30 gm. of body weight. Either 30 or 45 minutes later the animal was killed by a blow on the head, or guillotined, quickly opened, the small intestine removed, and washed out with ice-cold Ringer's solution. The lower fourth of the intestine was discarded, since it frequently contained considerable amounts of semisolid material, and since Verzár and Wirz (30) have reported that the upper half of the small intestine of the rat absorbs glucose more rapidly than the lower half. An ice-cold trichloroacetic acid filtrate of the mucosa[2] from the upper three-fourths of the intestine was prepared essentially as described by Laszt and Süllmann (9).

Phosphate Values Obtained for Trichloroacetic Acid Filtrates—The results are shown in Table II. Each phosphate value is an average value, and where enough tests were performed to make this possible the average value is followed by its standard error. Differences two or more times their standard error are considered significant. The phosphate values are expressed in mg. of phosphorus per 100 gm. of dried intestinal mucosa (mg. per cent, dry weight).

All the average values secured for four rats given 0.9 per cent NaCl plus 0.02 M phlorhizin plus 0.01 N NaOH are practically identical with those secured for eight control rats.

There is a considerable decrease in inorganic phosphate at 30 minutes and a still greater decrease at 45 minutes after glucose is given by stomach tube. As a mirror image of the change in inorganic phosphate, total organic phosphate is markedly increased at 30 minutes and still more increased at 45 minutes after the giving of the glucose solution. The decrease in inorganic phosphate and the increase in organic phosphate are much less if 0.02 M phlorhizin is mixed with the 5.5 per cent glucose solution.

Of the total increase of about 60 mg. per cent of organic P occurring in the mucosa of glucose-fed rats, about two-fifths was found in the 0 to 7

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[2] The intestine was slit longitudinally, and the mucosa scraped off and ground up in an iced mortar. A small portion was placed in Bottle 1 (previously weighed), the remainder in Bottle 2, containing 7.5 cc. of 15 per cent trichloroacetic acid, and also previously weighed. Mucosa and acid were thoroughly mixed and the two bottles reweighed. Bottle 1 was placed in a drying oven at 105° for 1 to 2 days before its final weighing. The contents of Bottle 2 were washed into a 25 cc. volumetric flask and ice-cold distilled water added to the mark. After 10 minutes vigorous shaking the contents of the flask were rapidly filtered through ash-free paper and placed immediately on ice.

TABLE II

Organic Phosphate Fractions in Intestinal Mucosa of Rats

The values in parentheses are average weight values.

Treatment	Body weight, gm.	No. of rats and sex	P values, mg. per cent dry weight, ± s.e.			Organic P fraction		
			Total P	Direct P	Total organic P	0–7 min. N HCl	7–180 min. N HCl	Difficultly hydrolyzable
Control experiments. Rats given nothing, or 0.9% NaCl by stomach tube								
	140–231 (165)	3 F., 5 M.	492.9 ±7.8	268.9 ±7.2	224.0 ±7.9	32.2 ±5.3	17.5 ±5.3	174.3 ±9.5
Phlorhizin controls. Rats given 0.9% NaCl + 0.02 M phlorhizin + 0.01 N NaOH by stomach tube								
	160–232 (190)	1 F., 3 M.	480.3	267.0	213.0	26.5	18.0	170.6
Rats given 5.5% glucose by stomach tube								
Killed, intestines removed 30 min. later	131–174 (158)	1 F., 4 M.	497.6 ±6.3	224.8 ±4.7	272.8 ±6.6	58.4 ±9.3	25.4 ±2.5	189.0 ±4.5
Intestines removed 45 min. later	158–203 (176)	2 F., 6 M.	493.9 ±10.7	199.4 ±10.3	294.5 ±9.2	54.9 ±3.3	32.9 ±4.5	206.6 ±7.7
Average.........	(169)	3 F., 10 M.	495.4 ±7.3	209.2 ±8.0	286.2 ±6.7	56.2 ±4.1	29.9 ±4.2	199.8 ±6.3
Rats given 5.5% glucose + 0.02 M phlorhizin + 0.01 N NaOH by stomach tube								
Intestines removed 30 min. later	146–215 (178)	5 M.	463.2 ±11.6	234.4 ±10.3	228.8 ±10.7	54.1 ±2.5	11.8 ±1.9	163.6 ±9.7
Intestines removed 45 min. later	157–210 (181)	1 F., 5 M.	507.0 ±16.9	249.3 ±12.5	257.7 ±11.0	52.6 ±4.1	17.7 ±2.9	187.5 ±9.9
Average.........	(180)	1 F., 10 M.	487.1 ±12.2	242.5 ±6.4	244.5 ±8.6	53.3 ±2.4	14.7 ±2.0	176.6 ±8.5
Statistically significant differences								
Values for glucose-fed minus control			−59.7 ±10.8	62.2 ±10.3	24.0 ±6.7	12.4 ±6.8		25.5 ±11.4
" " glucose + phlorhizin-fed minus control			−26.4 ±9.6	Not significant	21.1 ±5.8	Not significant		
Values for glucose-fed minus glucose + phlorhizin-fed			−33.3 ±10.2	41.7 ±10.9	Not significant	15.2 ±4.6		23.2 ±10.6

minute N HCl or pyrophosphate fraction, about one-fifth in the 7 to 180 minute N HCl fraction, and about two-fifths in the difficultly hydrolyzable fraction.

Rats fed glucose plus phlorhizin showed practically as great an increase in the pyrophosphate fraction as rats fed only glucose, but the phlorhizinized rats showed practically no change in organic P in either of the other two fractions. An examination of Table I shows that the carbohydrate-phosphate compounds which might be expected to accumulate in intestinal mucosa (hexose-6-phosphates, hexose diphosphate, and triose phosphates) after feeding of glucose would be found practically entirely in the 7 to 180 minute and the difficultly hydrolyzable organic P fractions. It is just here that phlorhizin exerts its inhibitory effects, while not affecting the accumulation of pyrophosphates, presumably formed from adenylic acid and inorganic phosphate.

Separation of Barium-Soluble and Barium-Insoluble Compounds in Trichloroacetic Acid Filtrates—The barium separation procedures were copied from Cori, Colowick, and Cori (2). 50 cc. or more of trichloroacetic acid filtrate of mucosa were prepared, two or more rats being used. 10 cc. of this filtrate were placed in each of two centrifuge tubes, together with 2 drops of 0.1 per cent phenolphthalein. Powdered barium hydroxide was added carefully, with stirring, until the contents of each tube showed a very faint pink. The precipitates which showed up at this point were centrifuged down, and the two supernatant fluids poured into a 25 cc. volumetric flask. The precipitates were redissolved as completely as possible with 0.01 N HCl, 2.0 cc. to each tube. 1 drop of phenolphthalein was added to each tube and powdered barium hydroxide worked in as before until the contents of each tube were a very faint pink. The precipitates were again centrifuged down, and the supernatant fluids added to those already present in the 25 cc. flask. This flask was filled to its mark with water and used for the determinations shown in Table III as Ba filtrate values.

The precipitates were almost completely dissolved with 1.0 N HCl, 1.0 cc. per tube, and the insoluble material centrifuged down and later discarded. The fluid portions were poured into a 10 cc. volumetric flask, which was brought to its mark with repeated water washings (and centrifugings) of the two tubes. This solution was employed to secure the determinations shown in Table III as Ba precipitate values.

The results of five experiments in which barium separation was combined with N HCl hydrolysis are shown in Table III. The conclusions which can be drawn from these results are as follows: (1) Where an increase in the 0 to 7 minute N HCl fraction occurred (compare Experiments B-1 and B-2 with A-1 and A-2) this increase took place largely in the barium

TABLE III

Combined Barium Separation and N HCl Hydrolysis Experiments

Solution tested	P values, mg. per 100 gm. dry weight				
	Total P	Direct P	Organic P fraction		
			0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.

Experiment A-1, Oct. 25, 1940, 2 rats (M. 170 gm., F. 175 gm.) fasted 24 hrs.

Solution tested	Total P	Direct P	0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.
Ba filtrate....	175	19	4	142	
" ppt...	206	196	10	3	1
Sum...	381	215	14	146	
Trichloroacetic acid filtrate..	510	284	24	41	161

Experiment A-2, Dec. 13, 1940, 2 female rats (177 gm., 172 gm.) fasted 24 hrs.

Solution tested	Total P	Direct P	0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.
Ba filtrate ..	263	66	20	11	166
" ppt.	186	148	16	7	15
Sum.	449	214	36	18	181
Trichloroacetic acid filtrate..	512	263	45	12	192

Experiment B-1, Nov. 27, 1940, 2 male rats (212 gm., 194 gm.) fasted, given 5.5% glucose by stomach tube, killed 45 min. later

Solution tested	Total P	Direct P	0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.
Ba filtrate... ..	264	35	22	41	166
" ppt.... ..	171	116	43	13	0
Sum.....	435	151	65	54	166
Trichloroacetic acid filtrate...	486	186	56	70	174

Experiment B-2, Jan. 3, 1941, 5 rats (M. 135 gm., 4 F. 149, 173, 151, 156 gm.) fasted, given glucose by stomach tube, killed 45 min. later

Solution tested	Total P	Direct P	0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.
Ba filtrate...	277	62	13	24	178
" ppt......	122	42	42	31	7
Sum....	399	104	55	55	185
Trichloroacetic acid filtrate....	444	135	61	53	195

Experiment C, Dec. 13, 1940, 2 female rats (191 gm., 182 gm.) given 5.5% glucose + 0.02 M phlorhizin + 0.01 N NaOH by stomach tube, killed 45 min. later

Solution tested	Total P	Direct P	0–7 min. N HCl	7–180 min. N HCl	P not hydrolyzed in 180 min.
Ba filtrate......	266	58	14	23	171
" ppt...........	191	157	21	13	0
Sum........	457	215	35	36	171
Trichloroacetic acid filtrate................	480	251	34	26	176

precipitate fraction, indicating formation of adenosine diphosphate or triphosphate or both. This is in accordance with the findings of Lohmann

(14) and of other workers on muscle and other tissues, and justifies the use of the term pyrophosphate fraction in referring to the 0 to 7 minute N HCl fraction of trichloroacetic acid filtrates of mucosa. (2) Failure to secure appreciable amounts of barium-soluble organic P, hydrolyzable within 7 minutes by N HCl at 100°, even after glucose feeding (again compare Experiments A-1 and A-2 with B-1 and B-2), indicates that only very small amounts of glucose-1-phosphate can be present in intestinal mucosa, and that if any is formed during glucose absorption it must be very rapidly

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TABLE IV

"Fructose" Content of Rat Intestinal Mucosa

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Type of experiment	"Fructose" concentrations, mg. per 100 gm. dried mucosa			
	Trichloroacetic acid filtrate	Ba filtrate fraction	Ba ppt. fraction	Ba filtrate + Ba ppt.
Control, Oct. 18, 1940...................	50.9	38.0	8.2	46.2
" Dec. 6, 1940...................	39.1	34.4	11.0	45.4
" " 19, 1940.................	23.7	16.6	3.8	20.4
Average for 9 experiments ± s.e......	34.7 ± 3.8			
Rats given glucose Nov. 27, 1940........	65.0	39.8	18.3	58.1
Jan. 3, 1941, mixed trichloroacetic acid filtrate, 5 rats........................	93.3	88.5	8.2	96.7
Average for 12 experiments ± s.e.....	92.0 ± 7.6			
Rats given glucose + phlorhizin Dec. 13, 1940..................................	56.2	54.2	3.2	57.4
Average for 7 experiments ± s.e.......	51.2 ± 3.9			

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Differences and s.e. for trichloroacetic acid filtrate averages

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Glucose value minus control.................................. 57.3 ± 8.5
"   + phlorhizin minus control.......................... 16.5 ± 5.4
"   minus glucose + phlorhizin........................ 40.8 ± 8.6

hydrolyzed or transformed. (3) Very little barium-insoluble difficultly hydrolyzable organic phosphate is present in rat intestinal mucosa. This indicates that only small amounts of phosphoglyceric acid can be present. It also suggests that any increase in difficultly hydrolyzable organic phosphate occurring after glucose feeding is probably due to formation of a hexose-6-phosphate; e.g., the Embden ester. This increase was noted after glucose feeding (Table II) but not after feeding glucose plus phlorhizin. (4) The difficultly hydrolyzable barium-soluble organic P

constitutes on the average over 60 per cent of the total organic phosphate and about one-third of the total acid-soluble phosphate. Fructose values (Table IV) indicate that the Embden ester constitutes only a minor part of this fraction.

"Fructose" in Trichloroacetic Acid and Derived Extracts of Rat Intestinal Mucosa

The Seliwanoff reaction (method of Roe (25)) gives strong color development with fructose (100 per cent), fructose-6-phosphate (about 60 per cent of that for fructose according to Goda (4)), and fructose diphosphate (about 25 per cent of that for fructose according to Kalckar (6)). In the present experiments the period of heating at 80° was changed from 8 minutes, as recommended by Roe, to 10 minutes, or 12 minutes, as this gave stronger color development and better checks between duplicates. All color determinations were made with a Coleman spectrophotometer set at either 490 or 500 mμ. Special tests were run on the comparative amounts of color secured with glucose and fructose standards. In terms of fructose, a fresh glucose solution gave a value of 0.69 per cent, while solutions which had been standing on ice for about 1 and 3 weeks respectively gave values of 0.78 and 0.93 per cent.

"Fructose" values secured for control rats, for rats given 5.5 per cent glucose and killed 45 minutes later, and for rats sacrificed 45 minutes after having been given glucose plus phlorhizin are shown in Table IV. The values shown in Table IV have been corrected for color development due to non-fructose-containing substances as follows: concentrations of reducing substances present in the filtrates were determined by the Hagedorn and Jensen method (23) and termed "total hexose." From the "total hexose" was subtracted the "fructose" hexose as determined by Roe's method (25). The difference was assumed to be glucose and multiplied by 0.0093 (the most unfavorable glucose to fructose ratio was 0.93 per cent). This value was then subtracted from the original "fructose" value. This correction amounted to only 2 to 4 mg. of "fructose" per 100 gm. of dried mucosa and was actually somewhat smaller for glucose-fed rats than for either control rats or rats fed glucose plus phlorhizin.

From Table IV it can be seen (1) that appreciable amounts of a compound or compounds giving the Seliwanoff fructose reaction are accumulated in the intestinal mucosa of glucose-fed rats; (2) that much smaller amounts of this compound or compounds are accumulated if phlorhizin is mixed with the glucose; (3) that much the greater part of the material reacting like fructose is barium-soluble; i.e., in so far as barium solubility is concerned it behaves like fructose-6-phosphate.

Values for the Embden ester in trichloroacetic acid filtrates may be

calculated from "fructose" values, if certain assumptions are made. For the present calculations it is assumed (a) that all of the material reacting like fructose is fructose-6-phosphate, (b) that fructose-6-phosphate gives 60 per cent of the color reaction given by fructose (Goda (4)), and (c) that this fructose-6-phosphate is present as part of the Embden equilibrium ester, which is composed of about 10 parts of fructose-6-phosphate to about 25 parts of glucose-6-phosphate.

$$\frac{\text{Hexose PO}_4\text{ P}}{\text{"Fructose"}} = \frac{100\%}{60\%} \times \frac{35(\text{total hexose PO}_4)}{10(\text{fructose PO}_4)} \times \frac{31.027(\text{mol. wt. of P})}{180.16(\text{mol. wt. of fructose})}$$

$$= 1.005$$

i.e., the hexose monophosphate P may be assumed to be identical with the "fructose" value. If we also assume that the increase in non-pyrophosphate P equals the increase in Embden ester P, we secure the following values for such increases, in rats killed 45 minutes after being given the test solution: rats fed glucose, (a) from increase in "fructose" value (Table IV) 57.3 mg. per cent of P, (b) from increase in non-pyrophosphate organic P (Table II) 47.7 mg. per cent of P; rats fed glucose plus phlorhizin, (a) from increase in "fructose" value (Table IV) 16.5 mg. per cent of P, (b) from increase in non-pyrophosphate organic P (Table II) 13.4 mg. per cent of P.

<div align="center">DISCUSSION</div>

Studies on tubular reabsorption of glucose by the kidney have led Shannon and Fisher (26) to postulate formation of addition compounds of glucose within the kidney cells to account for their results. The only compounds of this kind known at present to exist in kidney and intestinal mucosa cells, for formation and hydrolysis of which there exist powerful intracellular enzyme systems, are the carbohydrate-phosphate compounds.

If we assume that all of the increase in non-pyrophosphate organic P represents increase in hexose phosphate compounds or their successors, we get in 45 minutes an increase in concentration of these compounds in rat intestinal mucosa of 47.7 mg. per 100 gm. of dried mucosa (Table II) for rats absorbing 5.5 per cent glucose. In 45 minutes a 150 gm. rat may be expected to absorb about 0.25 gm. of glucose through an amount of intestinal mucosa having a total dry weight of about 0.4 gm. (unpublished observations). This amount of glucose is equivalent to over 10,000 mg. of hexose monophosphate P in 100 gm. of dried mucosa, or over 200 times the increase in non-pyrophosphate P found by analysis. As has already been pointed out by Verzár (27) and by others, the phosphorylation

hypothesis therefore requires that practically all of the hexose phosphate formed or some successor shall be almost immediately rehydrolyzed or shall diffuse out very rapidly into the blood capillaries of the small intestine.

Intestinal mucosa shows very considerable phosphatase activity, so that it is very probable that the splitting takes place chiefly in the mucosa. Whether any accumulation of carbohydrate-phosphate compounds shall occur in the mucosa will depend, then, not on the rate of phosphorylation of the hexose, but rather on the relative rates of phosphorylation and dephosphorylation.

Kjerulf-Jensen and Lundsgaard (7) have shown that, when cyanide is injected to stop intracellular phosphorylations without appreciably affecting phosphatase activities, a considerable part of the organic phosphate newly formed in rat intestinal mucosa during fructose absorption disappears very rapidly. They calculate that the rapidity of the observed phosphate splitting amounts to at least 95 per cent of the calculated rapidity of ester transformation when it is assumed that the ester formed during fructose absorption is fructose monophosphate.

Phosphorylation of glucose by kidney extracts is about 60 per cent inhibited by $M/600$ phlorhizin (5) and practically completely inhibited by $M/333$ phlorhizin (unpublished observations of the present author). Kidney phosphatase activity is only slightly affected between pH 6 and 9 by 0.01 M phlorhizin, and a concentration this great is required to bring about complete inhibition of the acid phosphatase at pH about 5 (further unpublished observations). In the kidney, then, phosphorylation of glucose is far more strongly inhibited by phlorhizin than is kidney phosphatase activity at any pH conceivable as occurring within the living kidney cells. The phosphatase activities of intestinal mucosa extracts were even less sensitive to phlorhizin than those of kidney extracts; so that it seems likely that the same relationship holds for intestinal mucosa.

Failure to secure accumulation in intestinal mucosa of non-pyrophosphate organic P and of substances reacting like fructose in rats fed glucose plus phlorhizin is therefore attributed to a specific inhibition of glucose phosphorylation by phlorhizin. This in turn suggests very strongly that the simultaneously occurring inhibition of glucose absorption by phlorhizin is also due to an interference with glucose phosphorylation. For a final decision as to the validity of the phosphorylation hypothesis of hexose absorption, it seems to the present author that experiments must be performed in which labeled molecules are used. One series of such experiments has already been reported by Rapoport and coworkers (24), who found that turnover of pyrophosphate P in rat kidneys, as measured with the aid of radioactive P, is appreciably reduced by phlorhizin.

SUMMARY

1. After 30 to 45 minutes absorption of a 5.5 per cent glucose solution the intestinal mucosa of the rat showed an average increase in pyrophosphate organic P of 24.0 mg. per cent (mg. per 100 gm. of dried mucosa), and average increases of 12.4 and 25.5 mg. per cent in the 7 to 180 minute N HCl and the difficultly hydrolyzable fractions respectively.

2. Barium separation procedures and "fructose" determinations indicated that most of the increase in non-pyrophosphate organic P was due to accumulation of a hexose monophosphate ester, probably the Embden equilibrium ester.

3. Increases in the non-pyrophosphate organic P fractions and in compounds reacting like fructose were much smaller in rats fed glucose plus phlorhizin than they were in rats fed only glucose.

4. Increases in pyrophosphate organic P were of about the same value in glucose-fed and in glucose plus phlorhizin-fed rats.

5. The relation between these findings and the phosphorylation hypothesis of glucose absorption by intestinal mucosa has been discussed.

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STUDIES ON MANGANESE DEFICIENCY IN THE RAT[*]

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Manganese has been shown by a number of investigators to be an essential nutritional element (1–9). Some discrepancy exists as to the effects of manganese deficiency. The estrous cycle has been reported to be both normal (4) and disturbed (1, 2, 5). Kemmerer, Elvehjem, and Hart (2) have reported that manganese deficiency retards growth in mice, while Orent and McCollum (4) have reported that rat growth is not affected by manganese deficiency.

In reference to the mechanism of manganese function little is understood. Rudra (10, 11) has reported that manganese is essential for the synthesis of ascorbic acid. This is of interest, as ascorbic acid has been used successfully for treating bovine sterility (12). Manganese may also play a rôle in certain enzyme systems. Richards and Hellerman (13) and Edlbacher and Baur (14) have reported that manganese may be a component of arginase. Manganese activation of certain dipeptidases has been shown by Berger and Johnson (15) and Gailey and Johnson (16).

These investigations were undertaken to determine whether any relation existed between manganese and ascorbic acid synthesis, to measure the effect of manganese deficiency on certain enzyme systems, and to make histological examination of various tissues of the manganese-deficient rat. At the same time it was hoped to produce a pronounced manganese deficiency and thereby further elucidate the effects of manganese on growth and reproductive functions.

EXPERIMENTAL AND RESULTS

In the first experiment both mineralized milk (1 mg. of iron and 0.1 mg. of copper per 100 cc. of milk) and a solid ration, composed of sucrose 63 parts, reprecipitated casein 20 parts, butter fat 6 parts, salts[1] 5 parts, yeast 5.9 parts, and irradiated yeast 0.1 part, were used for the low manganese rations. The milk ration contained about 0.03 part per million and the dry ration about 2 p.p.m. of manganese as determined by the

[1] The salt mixture of Phillips and Hart (17) with the manganese omitted was used.

method of Wiese and Johnson (18). Rats for these and subsequent experiments were weaned without access to manganese by placing the pregnant female on the low manganese ration during the last few days of gestation and during the suckling period. Glass cages were used, with absorbent cotton as bedding for the young. That this technique produced young rats with low stores of manganese was indicated by the poor growth subsequently obtained on the low manganese diets. Manganese supplementations, when given, were added so as to make the concentration of the milk 10 p.p.m., and the dry ration 50 p.p.m.

Four groups of six rats each were used in the first experiments; one group received mineralized milk, one group the dry low manganese ration, and two groups the same rations supplemented with manganese. The unsupplemented rats on both rations showed retardation of growth. However, growth in all the lots was only fair, due possibly to the unthrifty condition of the rats at weaning. After 10 weeks on experiment the rats were removed, and the tissues used for ascorbic acid analyses and histological studies. Since the rats reared on the mineralized milk had shown the most pronounced growth difference, mineralized milk was used for further studies. Three groups of three male and four female rats each were used. One lot received no supplement, one added ascorbic acid, and one added manganese. Ascorbic acid equivalent to 10 mg. per rat per day was given, either by subcutaneous injection or in the milk. Ascorbic acid additions to milk were made every other day, alternating with the mineral additions. The ascorbic acid supplements were discontinued at 30 weeks, as it had become evident that they gave no growth stimulation to manganese-deficient animals. After the rats had been on experiment for 26 weeks, vaginal smears were made on the females to determine the normality of the estrous cycles, and mating tests with stock colony male and female rats were made. After 48 to 52 weeks on experiment the rats were removed, and the tissues used for the enzyme studies and bone examinations.

Growth and Reproductive Functions—A definite growth response to manganese was obtained in both male and female rats, as shown in Figs. 1 and 2. It was hoped that by maintaining the rats on experiment for extended periods more pronounced effects of manganese deficiency could be produced but this was not realized. The growth difference lessened somewhat after 30 weeks. This may have been due to manganese contamination in the milk, or to other contamination as the rats at this time were transferred from glass to metal cages.

Food consumption records for the first 14 weeks of the second experiment showed that the groups on low manganese and low manganese plus ascorbic acid consumed an average of 41 and 37 cc. of milk per day re-
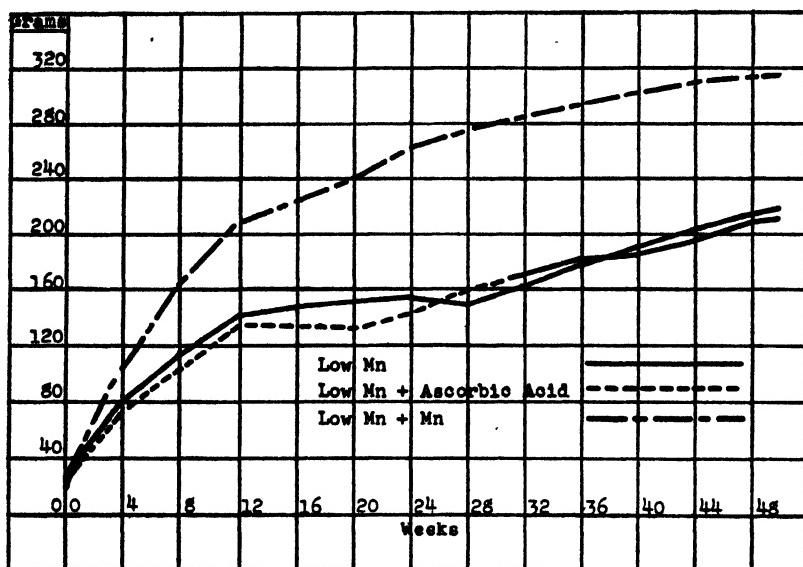
FIG. 1. Growth of male rats on low manganese diet
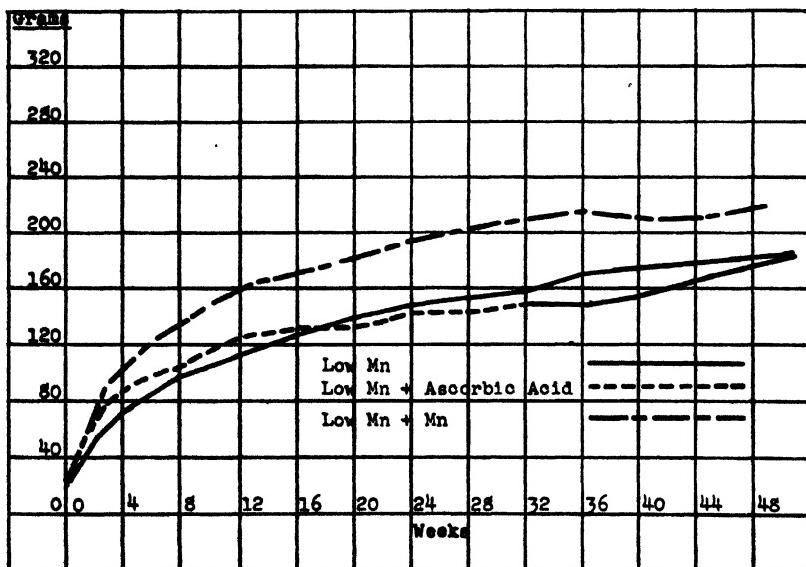
FIG. 2. Growth of female rats on low manganese diet

spectively, while the animals on the manganese-supplemented diet consumed an average of 50 cc. per day during this period.   The growth obtained per

100 cc. of milk consumed during the 14 week period was 2.32 gm. for the low manganese group, 2.34 gm. for the low manganese plus ascorbic acid group, and 2.84 gm. for the low manganese plus manganese group. This indicated a greater efficiency in the use of food by the rats receiving manganese supplements.

The females on the low manganese diet showed a marked delay in the opening of the vaginal orifice, the average time of opening being 171 days as compared to 82 days for controls. Estrous cycles as determined by vaginal smear technique in the manganese-deficient females were either irregular or absent, while control females showed with few exceptions regular estrous cycles. In breeding tests control females bred with stock colony males, but manganese-deficient females would not breed with stock colony males. Control males successfully bred stock colony females, but manganese-deficient males showed no interest in stock colony females.

TABLE I

Ascorbic Acid Content of Various Tissues in Manganese-Deficient Rat

Tissue	Ascorbic acid per gm. fresh tissue			
	Dry ration		Milk ration	
	Low manganese	Added manganese	Low manganese	Added manganese
	mg.	*mg.*	*mg.*	*mg.*
Kidney	0.12	0.12	0.11	0.10
Liver.	0.16	0.18	0.23	0.25
Adrenal.	2.8	3.1	3.3	3.5
Testes.	0.29	0.22	0.26	0.21

As to general symptoms the low manganese rats showed no gross symptoms other than a poorer hair coat and some alopecia around the neck. Diarrhea and coprophagy were common. The rats showed no impairment of gait, and were lively and active.

Relation to Ascorbic Acid—A manganese deficiency did not result in a lowered ascorbic acid content of the various tissues studied, as shown in Table I. The ascorbic acid content was measured by weighing about 200 mg. of the fresh tissue into 5 cc. of 6 per cent metaphosphoric acid, grinding with sand, making up to 10 cc. volume, filtering, and determining the ascorbic acid content of a suitable aliquot by use of the photoelectric colorimeter, as in the method of Mindlin and Butler (19). The lack of a growth response to ascorbic acid supplementation by the manganese-deficient rat (Figs. 1 and 2) was additional indication that ascorbic acid played no part in manganese deficiency. To check further any possible relationships of manganese to vitamin C, the experiments of Rudra (10)

were repeated. Rudra reported that rat liver slices synthesized vitamin C from mannose, and that manganese was a limiting factor in the synthesis. Duplication of Rudra's technique with liver, and also with kidney, intestine, and spleen, showed no synthesis of ascorbic acid from mannose, and no activating influence of manganese, as indicated in Table II. These experiments were also tried with an atmosphere of nitrogen, and with use of blood serum instead of Ringer-Locke solution and phosphate buffer as a suspending medium, but no synthesis could be detected. In all cases a loss of vitamin C occurred upon incubation.

Histological Studies—Histological examinations were made on the liver, kidney, adrenal, pituitary, thyroid, and testes or ovary. They revealed no marked abnormalities in any of the tissues except the testes. The livers of four out of six rats on the low manganese, dry ration showed some

TABLE II

Possible Synthesis of Ascorbic Acid by Rat Tissue

About 400 mg. of tissue slices in 3.0 cc. of Ringer-Locke solution and 2.0 cc. of 0.05 M phosphate buffer, pH 7.4; total volume 4.0 cc.; incubated 3 hours at 37°.

The results are reported in mg. of ascorbic acid per gm. of fresh tissue.

Additions	Before incubation				After incubation			
	Liver	Kidney	In-testine	Spleen	Liver	Kidney	In-testine	Spleen
None	0.45	0.27	0.30	0.56	0.26	0.13	0.10	0.42
20 mg. mannose.					0.26	0.15	0.10	0.50
Mn, final concentration 0.002%					0.27	0.11	0.03	0.42
Mannose and Mn					0.25	0.09	0.05	0.39

vacuolar degeneration. One out of six rats on the dry ration with added manganese was similarly affected. The testes of the manganese-deficient rats all showed degeneration. There was a complete lack of spermatids and spermatozoa. Nuclear division was decreased or absent, which resulted in a decrease in the size of the individual tubules. The epithelium of the epididymus was fairly normal, although the tubules were materially decreased in size. The lumen of the tubules was filled with degenerating cells instead of spermatozoa.

Enzyme Studies—The activity of certain intestinal dipeptidases was studied by means of thirteen deficient and five control animals. The entire small intestine was removed, the length determined, and the upper 25 per cent taken for study. The contents were removed by gentle squeezing, the tissue weighed, homogenized by grinding with sand and 50 per cent glycerol, and made to 10 cc. volume with 50 per cent glycerol. The ac-

tivity of these preparations in splitting glycylglycine and leucylglycine
was determined on suitable aliquots, with and without additional man-
ganese as an activator, according to the methods used by Berger and John-
son (15). No deficiency of dipeptidase activity in splitting the substrates
used was evident in the manganese-deficient animal. The dipeptidase
activity showed a fairly wide variation, but averages for dipeptidase
activity of intestinal preparations from the manganese-deficient animals
were equal to or slightly greater than those for the controls.

Arginase studies on liver tissue were made by adapting the method used
by Lightbody (20). About 200 mg. of fresh liver tissue were homogenized
with distilled H_2O and made to 25 cc. volume. 4 cc. of resulting suspension
and 1.0 cc. of veronal buffer, pH 9, (21) were made to 10 cc. volume, and
placed in a water bath at 37° for 1 hour. Then 0.5 cc. of the enzyme
preparation was pipetted into a test-tube containing 2 cc. of 1 per cent
arginine previously brought to pH 9, 1 cc. of veronal buffer (pH 9), and
1.5 cc. of H_2O, or 1.0 cc. of H_2O, and 0.5 cc. of 0.0015 M $MnCl_2$. To the
control tubes were added immediately 2 drops of 1:1 HCl, and the tubes
were placed in a boiling water bath for about 5 minutes. Samples were
incubated at 37° for 2 hours, then acidified, and heated. The urea liber-
ated was determined by bringing the samples to approximately pH 5 with
1 N NaOH, brom-cresol green being used as an indicator. 1 cc. of 3 N
acetate buffer (pH 5.0) and 1 cc. of freshly prepared urease solution were
added; then samples were incubated at 37° for 30 minutes. The ammonia
liberated was determined by washing the sample into an aeration tube
containing 5 cc. of saturated K_2CO_3, and aerating for 2 hours into 10 cc.
of 0.01 N HCl. The HCl was titrated with 0.01 N NaOH with chlor-
phenol red as the indicator. The urease solution used was prepared by
making 200 mg. of Squibb's urease, 1 cc. of acetate buffer (pH 5), and 1
cc. of 0.1 M NaCN to 10 cc. volume.

In these studies doubling the amount of enzyme preparation resulted in
slightly less than a 2-fold increase in activity. This necessitated de-
termining the amount of urea liberated at different enzyme concentrations,
and subsequently expressing results in terms of a definite concentration of
enzyme. For these studies a "unit" of activity was taken as that amount
of enzyme which under the conditions of the determination would liberate
10 mM of urea. A lowered liver arginase was found in the manganese-
deficient rat, as indicated in Table III. Results are expressed in terms of
the weight of fresh liver tissue, since the dry weight of the livers showed no
essential difference between deficient and control animals.

The liver arginase from the animals which had received manganese as
well as that of the manganese-deficient animals showed activation with
added manganese under the conditions of the determination. The amount

of manganese added was found to be sufficient for maximum activation of preparations from both the deficient and the control animals, indicating that the differences in arginase activity found represented differences of enzyme concentration and not of activation.

Preliminary studies on urinary nitrogen excretion with two deficient rats, which remained after the arginase studies, showed only a slight decrease in the per cent of total urinary nitrogen excreted as urea, and no modification of the per cent excreted as ammonia, amino acid, and uric acid nitrogen. However, as these results were obtained from old rats

TABLE III

Concentration of Liver Arginase in Manganese Deficiency

The results are reported in units* of arginase per 100 mg. of fresh liver.

Deficient animals		Control animals	
No added manganese	Added manganese	No added manganese	Added manganese
25	65	88	218
32	84	81	175
71	97	58	130
43	86	87	133
66	85	74†	
65	91	111†	
57	101	80†	
35	46		
48	96		
60	87		
59	103		
42	58		
61	79		
58			
52			
Average..52	83	83	164

* A unit of activity is that amount of enzyme which, under the conditions of the determination, would liberate 10 mM of urea.

† Stock rats.

after much of the earlier growth difference had disappeared, further studies are indicated.

In relation to other possible rôles of manganese no bone abnormalities such as those reported in two young rats by Barnes, Sperling, and Maynard (9) were found with these older rats. They reported that the width of the tibia at the proximal end was large in proportion to the length of the bone. In the rats from these experiments the ratio of the width of the tibia at the widest point of the proximal end to the length of the tibia averaged 0.17 for both deficient and control animals.

DISCUSSION

The results of these experiments demonstrate that manganese deficiency can result in marked growth retardation in the rat. Other unpublished data from this laboratory have shown deficient growth on low manganese rations.[2] The failure of Orent and McCollum (4) to show retarded growth on milk diets or other diets low in manganese may have been due to manganese stores in the rats when placed on experiment, as these investigators used stock colony young for their experiments. The importance of using rats with low manganese stores is shown in these experiments.

Our results confirm those of other investigations which showed that a manganese deficiency disturbs the estrous cycle of the rat (1, 5) and mouse (2). The normal estrous cycle reported by Orent and McCollum (4) for the manganese-deficient female rat was probably due to lack of a sufficient degree of manganese deficiency. The delay in the opening of the vaginal orifice found in these experiments is greater than that reported by Skinner et al. (5).

The results of these studies indicate that manganese is not necessary for the synthesis of ascorbic acid. Guha and Gosh (22) first reported that various rat tissues could synthesize ascorbic acid from mannose, but Hawthorne and Harrison (23) and Klodt (24) could not repeat the results of Guha and Gosh. Rudra (10) then reported that the limiting factor in the synthesis was manganese, and has later reported that guinea pig as well as rat liver could synthesize vitamin C from mannose in the presence of manganese (11). Our attempts to repeat Rudra's work have been unsuccessful. They do substantiate the results of Hawthorne and Harrison (23) and Klodt (24).

The significance of the arginase differences found is not clear. That milk diets on which these enzyme differences were found are not entirely adequate is well known (4, 6, 25). However, the differences noted in the arginase concentration can be attributed to the added manganese, as it was the only dietary difference in two groups.

SUMMARY

Pronounced manganese deficiency in the rat has been produced by use of rats weaned without access to manganese. This deficiency resulted in definitely impaired growth in the male and the female rat.

In the manganese-deficient female rat estrous cycles were irregular or absent, and there was a marked delay in the opening of the vaginal orifice. A manganese deficiency in the male rat caused testicular degeneration and complete sterility due to lack of spermatozoa production. Both male and

[2] Wachtel, L. W., and Hart, E. B., unpublished data.

female manganese-deficient rats were unable to reproduce. No histological abnormalities were detected in the adrenal, kidney, pituitary, and thyroid of the manganese-deficient rat.

The deficiency did not result in reduced ascorbic acid content of tissues, nor did ascorbic acid stimulate the growth of the manganese-deficient rat. Synthesis of ascorbic acid from mannose by rat liver and other tissues *in vitro* could not be obtained with or without added manganese.

A reduced arginase concentration in the liver of the manganese-deficient rat was found. There were no essential differences in the activity of the intestinal dipeptidases studied.

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THE VERATRINE ALKALOIDS

XIII. THE DEHYDROGENATION OF PROTOVERATRINE

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In our further study of the chemistry of the veratrine alkaloids it was of interest to apply to other alkaloids of *Veratrum album* methods similar to those which have been reported in the case of cevine and, more recently, of jervine (1). This has now been extended to the alkaloid protoveratrine, which was first isolated by Salzberger (2) and to which more recently the formula $C_{40}H_{63}O_{14}N$ has been given by Poethke (3). According to the latter this alkaloid yields on saponification acetic acid, *l*-methylethylacetic acid, methylethylglycolic acid, and an amorphous alkamine, protoverine, $C_{28}H_{45}O_{10}N$. Although a relationship in structure of this alkamine and its companion alkamine germine, $C_{26}H_{41}O_8N$ (3), to cevine, $C_{27}H_{43}O_8N$, has been suspected on general considerations, this has not as yet been supported by any direct evidence. Some such evidence we have now been able to obtain by a study of the dehydrogenation of protoveratrine.

Since the protoverine of Poethke is difficult to purify, we have dehydrogenated protoveratrine itself with selenium. The resulting reaction products yielded a large acid fraction from which it was possible to separate by fractional distillation acetic acid, methylethylacetic acid, and methylethylglycolic acid, thus confirming the observations on saponification by Poethke. From the volatile basic fraction it was possible to separate in relatively small amount a dimethylpyridine, which from the melting point of its picrate (171–174°) appeared to be 2,5-dimethylpyridine (4). A second volatile base was obtained in larger amount which agreed in properties both as the free base and as the picrate with the properties which we have recorded for the 2-ethyl-5-methylpyridine (5) obtained from cevine and from jervine. In a higher fraction an oxygen-containing base was contained which was isolated as the *picrate*. Analysis of the latter indicated a formulation $C_8H_9ON \cdot C_6H_3O_7N_3$. The undistilled residue which resulted from the dehydrogenation yielded a relatively small high boiling fraction. From the latter a crystalline fraction was in turn obtained but in an amount too small for recrystallization to constant melting point. However, analysis, absorption spectrum measurements, and general properties indicated impure cevanthrol, $C_{17}H_{16}O$. Its absorption spectrum curve,[1] together with that of cevanthrol, which hitherto has not been pub-

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[1] The absorption spectrum measurements were kindly carried out by Dr. G. I. Lavin of the Rockefeller Institute.

lished, is given in Fig. 1. It can readily be seen from the curves that the type of absorption is similar to that shown by cevanthrol, although the extinction coefficients are uniformly lower, a difference which may well have been due to impurity. Finally, from the non-volatile basic fraction a picrate was obtained in very small amount which when analyzed appeared to be cevanthridine picrate. Although the melting point was somewhat low, it gave no depression when mixed with cevanthridine picrate.

From these data there can be little doubt that the alkamine protoverine has the same type of ring structure as has cevine.
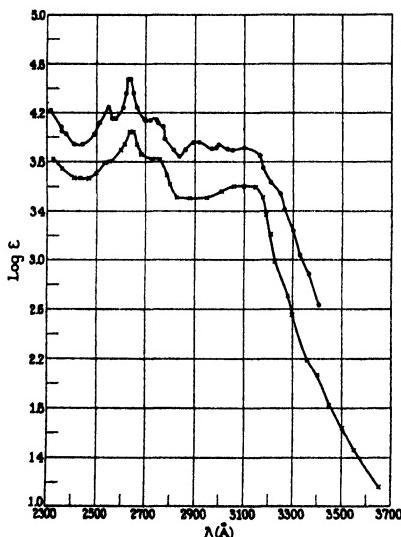
FIG. 1. Absorption spectrum curves determined in ethyl alcohol solution. ○ = cevanthrol; × = compound from protoveratrine.

EXPERIMENTAL

Protoveratrine—Ground commercial roots of *Veratrum album* were extracted at room temperature as follows: 2 kilo portions were thoroughly mixed with 7 liters of benzene and stirred with a mixture of 100 cc. of ammonia (sp. gr. 0.9) and 1 liter of H_2O. The next day the solvent was filtered and the moist solid was pressed dry. The latter was reextracted with an additional 5 liters of benzene. This was finally followed by a third extraction with 5 liters, which was used directly for the first extraction of a succeeding portion of root. The first two extracts, which had been obtained from a total of 8 kilos of roots, were concentrated *in vacuo* to 3 liters. The clear, dark colored solution was then extracted six times with

1 liter portions of 5 per cent acetic acid. The acid extracts were shaken with 2 liters of benzene and made alkaline with excess 25 per cent NaOH. The extraction with repeated portions of benzene was accomplished as quickly as possible to avoid crystallization of the alkaloid. The benzene extract was washed with water several times, during which a small amount of sparingly soluble alkaloid material (protoveratridine) remained suspended in the aqueous phase. The benzene layer was dried and concentrated to dryness. The resinous residue was dissolved in about 500 cc. of dry ether. Crystallization promptly occurred, and after 18 hours the solid was collected with ether. The yield of this fraction from 8 kilos of root averaged 9.2 gm. This was followed soon by a second fraction from the mother liquor which averaged nearly 4 gm. After many weeks an appreciable third fraction of more soluble alkaloids gradually crystallized. The first fraction consisted essentially of protoveratrine.

For recrystallization the first fraction was suspended in about 10 parts of hot 95 per cent alcohol and an excess of acetic acid was added. On addition of a slight excess of ammonia the alkaloid rapidly separated as a crystalline powder which discolored above 270° and gradually decomposed at 273–276° (uncorrected). On concentration of the mother liquor *in vacuo* a second appreciable crop was obtained.

$$[\alpha]_D^{27} = -40° \ (c = 1.03 \text{ in pyridine})$$
$$C_{40}H_{63}O_{14}N. \quad \text{Calculated.} \quad \text{C } 61.42, \text{ H } 8.13$$
$$\text{Found. } (a) \quad \text{`` } 61.64, \text{ `` } 8.36$$
$$\text{`` } \quad (b) \quad \text{`` } 61.37, \text{ `` } 8.37$$

Dehydrogenation of Protoveratrine—A mixture of 16.6 gm. of protoveratrine and 60 gm. of selenium was heated in a flask after the air was replaced by nitrogen at 340° for 2 hours. 10.5 gm. of oil distilled over into a chilled receiver. The distillate was treated with a little more than 1 equivalent of 1:1 HCl (on the basis of the original alkaloid) and extracted with ether. The ether layer which contained acid and neutral material was set aside to be treated as described below. The aqueous layer was made strongly alkaline with solid KOH and the liberated bases were extracted with a little ether. Treatment of the alkaline aqueous layer with CO_2 did not liberate any phenolic material.

The ether solution of bases was dried over K_2CO_3, concentrated somewhat, and placed in a fractionation apparatus with a fractionating column 10 cm. in length. The record of the fractionation is given in Table I. Each fraction contained approximately 60 mg. of oil.

The analytical data of Fraction 1 suggested a picoline. However, a picrate crystallized readily from acetone and gave analytical data which suggested a dimethylpyridine. After two recrystallizations from acetone

yellow needles were obtained which melted at 171–174°.   2,5-Dimethyl-pyridine picrate has been reported to melt at this point (4).

$C_7H_9N \cdot C_6H_3O_7N_3$.   Calculated, C 46.41, H 3.60; found, C 46.65, H 3.51

Fractions 3, 4, 5, and 6 did not show much variation in boiling point or refractive index.   Both were in good agreement with the figures for 2-ethyl-5-methylpyridine obtained from the zinc dust distillation of cevine (5).   The analytical data of Fraction 5 also approached the calculated figures for $C_8H_{11}N$; *viz.*, C 79.27, H 9.15.   It formed a picrate from acetone solution which melted sharply at 144–145° and agreed in all properties with the picrate of 2-ethyl-5-methylpyridine.   The mixture showed no melting point depression.

$C_8H_{11}N \cdot C_6H_3O_7N$.   Calculated, C 47.98, H 4.03; found, C 48.35, H 4.06

TABLE I

Fractionation of Volatile Bases

Fraction No.	Bath tempera-ture	Column tempera-ture	Pressure	Micro b.p.	n_D^{25}	Analysis	
						C	H
	°C.	°C.	mm.	°C.		per cent	per cent
1	95	63	40	147	1.4942	76.68	7.81
2	95	65	36	156	1.4952		
3	95	67	29	167	1.4952		
4	97	68	29	171	1.4950		
5	97	65	20	171	1.4950	78.88	9.15
6	103	65	10	173	1.4956		
7	170	130	10	202	1.5152		
8	200	130	2	220	1.5245	70.50	8.05

Fraction 8 with 40 mg. of picric acid yielded 12 mg. of compact rhombic crystals from acetone which melted at 114–117°.

$C_8H_9ON \cdot C_6H_3O_7N_3$.   Calculated, C 46.14, H 3.30; found, C 46.14, H 3.53

Recrystallization of this material gave crystals which melted at 138–145°. This result combined with the analytical data of Fraction 8 itself makes the experience with this alkaloid similar to that encountered with cevine. Although the analytical data of Fraction 8 indicate a formulation of $C_8H_{11}ON$ (calculated, C 70.02, H 8.08), the picrate prepared from it indicates the presence of a small fraction containing less hydrogen.   In our previous experience with cevine it was found that a crystalline picrate could not be obtained from the $C_8H_{11}ON$ base (6).

The above ether extract, which contained the acid and neutral fractions, was in turn extracted with NaOH solution.   The neutral fraction which remained in the ether did not yield anything of promise and appeared to

consist mostly of selenium derivatives. The aqueous alkaline extract was saturated with CO_2 and investigated for any phenolic products. This proved to be negative. The aqueous layer was then acidified with an excess of dilute HCl and exhaustively extracted with ether. The combined ether extract after drying over $MgSO_4$ was concentrated under a fractionating column. An aliquot of the oily residue of mixed acids was fractionated in a fractionating apparatus with a 10 cm. column, as given in Table II. Each fraction contained approximately 100 mg. Fraction 13 was crystalline.

Fraction 3 was found to be practically pure acetic acid ($C_2H_4O_2$, calculated, C 39.99, H 6.70). The boiling point, melting point, and refractive

TABLE II

Fractionation of Acidic Volatile Material

The temperature of the bath was 100° in all cases.

Fraction No.	Column temperature	Pressure	Micro b.p. (760 mm)	M.p.	n_D^{25}	Analysis	
						C	H
	°C.	mm.	°C.	°C.		per cent	per cent
1	55	120	115				
2	65	100	115.5	14–15.5			
3	65	70	116	15–16	1.3728	40.00	6.73
4	65	33	118				
5	65	30	124				
6	65	24	157				
7	65	21	173				
8	65	19	174				
9	65	19	174				
10	65	18	174		1.4059	58.80	9.75
11	65	15	174				
12	65	5	174				
13	65	0.2					

index were also in excellent agreement. Fractions 1, 2, and 4, from the data above, appeared to consist essentially of this acid.

The boiling points of Fractions 7 to 12 closely approximated the constant of isovaleric acid. Their identity was substantiated by the refractive index (of Fraction 10), smell, and the analytical data ($C_5H_{10}O_2$, calculated, C 58.77, H 9.87).

The final fraction (No. 13) was crystalline but still contained a little oil. However, the crystals did not entirely melt until a temperature of 68° was reached. Upon recrystallization from a mixture of ether and isopentane felted needles were obtained which melted at 71–73° (Poethke reported 72.5–73°).

$C_5H_{10}O_3$. Calculated, C 50.81, H 8.54; found, C 51.12, H 8.45

The non-volatile residue from the dehydrogenation was powdered and exhaustively extracted with ether. Evaporation of the combined ether extracts gave a residue of only 0.6 gm. It was redissolved in ether and extracted with 10 per cent HCl. The ether layer was separated from the usual tarry precipitate and dried. The acid layer was set aside to be treated as described below. The ether solution yielded a residue of 0.2 gm. For fractionation it was placed in a micro fractionating apparatus with a column 5 cm. in length. Several small fractions were collected up to an oil bath temperature of 230° and under 5 mm. pressure. They appeared, however, to be of hydrocarbon character and were not sufficient in amount to permit of proper fractionation. However, a later fraction of 50 mg. of semicrystalline material contaminated with selenium distilled up to 240°. The selenium was removed with bone-black in ether solution. The filtrate yielded a residue which was dissolved in a little benzene. Upon cooling and seeding with cevanthrol 23 mg. of crystalline material separated which did not exhibit a sharp melting point. After recrystallization 11 mg. of leaflets were obtained which melted at 168–175°.

$C_{17}H_{16}O$. Calculated, C 86.39, H 6.83; found, C 85.92, H 7.29

Obviously the analytical data and melting point are not entirely satisfactory (cevanthrol melts at 195–196°), but there was not sufficient material for further recrystallization after the analysis and absorption spectra measurements.

The above acid layer with the suspended tarry salts of basic products was extracted with chloroform. The chloroform, which dissolved all the precipitated tar, was extracted with 10 per cent NaOH and dried over K_2CO_3. Evaporation of the extract gave a residue of 250 mg. Upon distillation under 0.2 mm. pressure only a trace distilled up to an oil bath temperature of 200°. However, above this up to 255°, 130 mg. of distillate were collected. This could not be directly crystallized. When mixed with 70 mg. of picric acid in acetone solution, 20 mg. of crystalline material were obtained which melted at 235–245°. This melting point was not raised on recrystallization and is approximately 5° lower than that of cevanthridine picrate. A mixed melting point, however, showed no depression.

$C_{25}H_{27}N \cdot C_6H_3O_7N$. Calculated, C 65.24, H 5.30; found, C 64.92, H 5.56

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THE DISSOCIATION CONSTANTS OF HYDROXYLYSINE

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The position of the hydroxy group in the amino acid hydroxylysine, whose isolation was reported in 1938 (1), has not been definitely established.

The chemical evidence points to one of two positions as indicated by Formulas I and II.

$$CH_2NH$$
$$|$$
$$CHOH$$
$$|$$
$$(CH_2)_2$$
$$|$$
$$CHNH_2$$
$$|$$
$$COOH$$
$$(I)$$

$$CH_2OH$$
$$|$$
$$CHNH_2$$
$$|$$
$$(CH_2)_2$$
$$|$$
$$CHNH_2$$
$$|$$
$$COOH$$
$$(II)$$
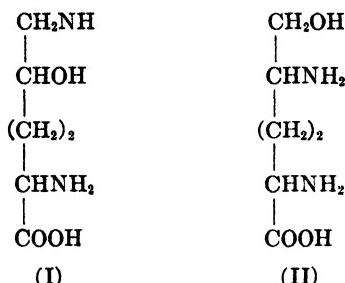
It is the purpose of the present communication to present values of the dissociation constants of the acidic and basic groups of hydroxylysine, and to compare these values with the corresponding constants of lysine. The influence on the acid-base dissociation constants of the introduction of the hydroxy group into the lysine molecule provides additional evidence for the correctness of Formula I or II.

Material

The hydroxylysine used was a preparation of the crystallized monochloride prepared from gelatin by Van Slyke, Hiller, Dillon, and Mac-Fadyen (1). On analysis, the yields were as follows:

$C_6H_{14}O_3N_2 \cdot HCl$. Calculated. C 36.27, H 7.61, N 14.11, NH_2-N 14.11, Cl 17.85
Found. " 36.29, " 7.72, " 14.12, " 13.95, " 18.23

For the carbon, hydrogen, and nitrogen values, by Pregl micro combustion, we thank Dr. A. Elek. The amino nitrogen was determined by the manometric nitrous acid method (2). The chloride content was by the microtitration of Sendroy (3).

Methods

The dissociation constants were calculated from the titration curves of lysine and hydroxylysine.

The nature and scarcity of the material used made certain adaptations of the conventional technique necessary. Since rather strongly acid and alkaline dissociation constants had to be determined, dilution of the amino acid could not be carried below a molarity of 0.01 without a sacrifice of accuracy. Therefore, a small titration vessel (Fig. 1) was constructed from a 25 × 120 mm. centrifuge tube, to which were fused inlet and outlet tubes for hydrogen. The rubber stopper of the vessel carried two platinized platinum electrodes (*a*) (or in some experiments also a bulb type glass
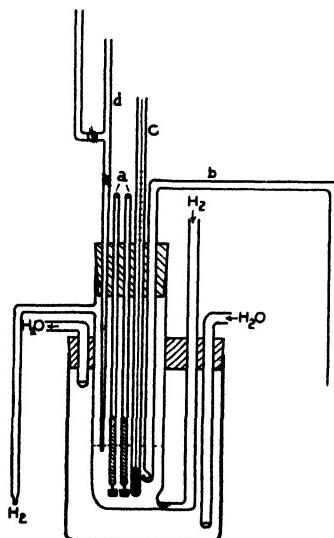
FIG. 1. Microtitration vessel

electrode), a KCl agar bridge (*b*) connecting to a saturated calomel cell, a thermometer (*c*), and an automatic 1 cc. micro burette (*d*), the tip of which was finely drawn out and immersed in the solution. The titration vessel was surrounded by a jacket through which water at 38° was circulated. This assembly was kept in an electrically shielded air thermostat at 38°. The potentials of the hydrogen electrodes were measured with a Leeds and Northrup type K potentiometer and type R galvanometer. When pH values were measured with the glass electrode, potentials were taken with an additional vacuum tube amplifier which was included in the potentiometric circuit.

3 to 4 mg. of hydroxylysine monohydrochloride were weighed out on the micro balance and dissolved in 10 cc. of 0.1 N KCl. After equilibration

with hydrogen, the solution was titrated successively with 0.1 N HCl and 0.1 N NaOH. Titrations of lysine dihydrochloride were performed with 0.1 N NaOH, followed by back titration with acid.

All pH measurements were referred to standard acetate buffer to which was assigned a value of pH 4.65 according to Hitchcock and Taylor (4).

The results were calculated according to Van Slyke's equation (5)

$$K = \frac{[H^+] \times ([B] + [H^+] - [OH^-])}{C - ([B] + [H] - [OH^-])}$$

TABLE I
Titration of Hydroxylysine Monohydrochloride

C = the total concentration of ampholyte; B = the concentration of NaOH. A negative value of B designates the concentration of HCl.

pH	C	B	pK'₁	pK'₂	pK'₃
	mм per l.	*mм per l.*			
2.34	1.80	−0.76	2.18		
2.49	1.77	−0.54	2.12		
2.59	1.76	−0.44	2.11		
2.72	1.75	−0.33	2.12		
2.90	1.73	−0.22	2.25		
3.08	1.72	−0.15	2.17		
8.42	1.69	0.75		8 63	
8.55	1.68	0.90		8.65	
8.74	1.68	1.14		8.67	
8.86	1.68	1.30		8.65	9.65
9.03	1.67	1.54		8.64	9.66
9.19	1.67	1.78		8.64	9.67
9.35	1.67	2.02		8.66	9.64
9.56	1.66	2.34		8.65	9.66
9.72	1.66	2.58			9.69
9.88	1.65	2.81			9.70
10.03	1.65	3.12			9.66

where K = the dissociation constant, [B] = the concentration of NaOH added; it assumes a negative value when HCl was added to the solution. C = the total concentration of ampholyte. The value of $[H^+]$ was calculated from $\alpha_H \times 0.84$ where $\alpha_H = 10^{-pH}$ and 0.84 was the activity coefficient of $[H^+]$ under the conditions of our experiment. Similarly, the value of $[OH^-]$ was calculated from $\alpha_{OH} \times 0.81$ where $\alpha_{OH} = 10^{13.55-pH}$ and 0.81 was the activity coefficient of $[OH^-]$. The value of 13.55 for pK_w was determined experimentally by measuring pH values of various concentrations of NaOH in 0.1 N KCl under conditions identical with those of the experiment. From these data, it was calculated according to the equation, $pK_w = pH - \log [NaOH] - \log 0.81$.

The dissociation constants of the two amino groups, the buffer actions of which overlap, were determined by successive approximation according to the procedure of Hastings and Van Slyke (6).

Results

The data from a representative titration of hydroxylysine are given in Table I and Fig. 2. In the acid range, the glass electrode and hydrogen elec-
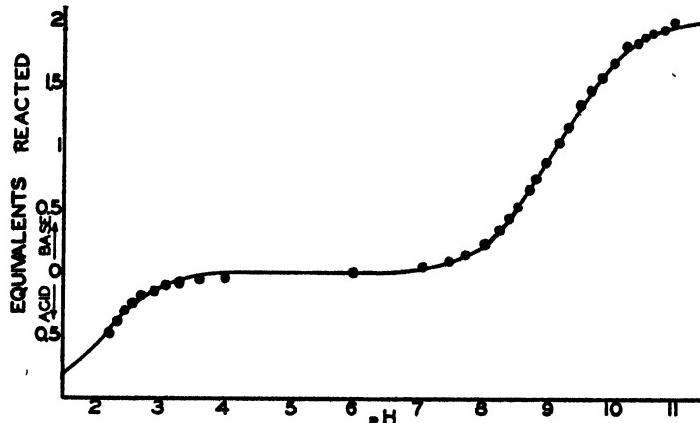
FIG. 2. Titration curve of hydroxylysine. The smooth curve is based on the constants, $pK'_1 = 2.13$, $pK'_2 = 8.62$, and $pK'_3 = 9.67$. The points are experimental.

TABLE II

Dissociation Constants of Hydroxylysine and Lysine

	pK'$_1$	pK'$_2$	pK'$_3$
Hydroxylysine	2.16	8.65	9.67
	2.22	8.63	9.66
	2.00	8.59	9.68
Average	2.13	8.62	9.67
Lysine	2.17	8.91	10.32
	2.23	8.90	10.24
Average.	2.20	8.90	10.28

trode pH values agreed within 0.01 pH; beyond pH 8, the deviation between the two methods became considerable. Although seven complete titrations of hydroxylysine were made, the data of only those titrations carried out at 38° with the hydrogen electrode will be reported. The values of the three dissociation constants, pK_1, pK_2, pK_3, corresponding to the carboxyl group and the first and second amino groups respectively, calculated from three experiments are given in Table II. The pK values of lysine, reported by

Schmidt, Kirk, and Appleman (7) as 2.18, 8.95, and 10.53 for 25° could not be used for comparison with our data on hydroxylysine because of the differences in temperature and ionic strength of the solutions. Lysine was, therefore, titrated under the conditions of our titrations of hydroxylysine. The results of these titrations are also given in Table II.

DISCUSSION

The introduction of an OH group into the lysine molecule does not significantly alter the pK' of the carboxylic group (pK'$_1$). It decreases the pK' of the α-amino group (pK'$_2$) by 0.28 unit. On the other hand, the dissociation constant of the second amino group of hydroxylysine is depressed from pK 10.28 to 9.67; i.e., 0.61 unit. From this, it would seem that the OH group is attached more closely to the second amino group than to the α-amino group, making its attachment to the δ-carbon the most likely position. Unfortunately, very few data are available about the influence of OH groups in various positions on an aliphatic amino group. A comparison with the dissociation constants[1] of ethylamine (pK' = 10.66) and ethanolamine (pK' = 9.48) shows that an OH group adjacent to the amino group may depress its pK by as much as 1.18 units.

However, it is shown by the dissociation constant of alanine (pK'$_2$ = 9.72) and serine (pK'$_2$ = 9.15) that the change in pK' of an amino group brought about by an adjacent OH group is smaller in an amino acid than in an otherwise unsubstituted aliphatic amine.

The dissociation constants of hydroxylysine are, therefore, consistent with Formulas I and II. The dissociation constants do not permit a differentiation between these two structures.

SUMMARY

The dissociation constants of hydroxylysine were determined at 38° as pK'$_1$ = 2.13, pK'$_2$ = 8.62, and pK'$_3$ = 9.67 as compared with those of lysine, pK'$_1$ = 2.20, pK'$_2$ = 8.90, and pK'$_3$ = 10.28. These data confirm the view that the hydroxy group is attached in the δ or ϵ position to the carbon atom adjacent to that carrying the amino group.

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[1] These values were taken from the review of Cohn (8).

THE REDUCTION OF METHEMOGLOBIN BY ASCORBIC ACID*

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In this laboratory a study of the oxygen capacities of chemically modified hemoglobins has recently been undertaken. In connection with this work the reduction of methemoglobin under carefully controlled conditions offered itself as a problem, since the combination of a ferriheme with globin leads to methemoglobin. In view of this consideration and of the recent reports of Cox and Wendel (1), Morrison and Williams (2), Gelinsky (3), and others on the reduction of methemoglobin *in vivo*, and those by Lemberg and coworkers (4, 5) on the coupled oxidation of ascorbic acid and hemoglobin, it was decided to make a study of the reaction between methemoglobin and ascorbic acid *in vitro*. This reaction is of particular interest, since it forms one step in the cycle postulated by Lemberg, Legge, and Lockwood (6) for the formation of choleglobin (and bile pigments).

It has been found in accordance with thermodynamic prediction that ascorbic acid at pH 7 and 0° will reduce methemoglobin to the extent of 80 to 90 per cent within 6 hours. When the reaction is carried out at room temperature in the presence of methylene blue, interaction between the two systems is complete at the end of 1 hour.

The accompanying reactions have been studied by the oxygen capacity method.
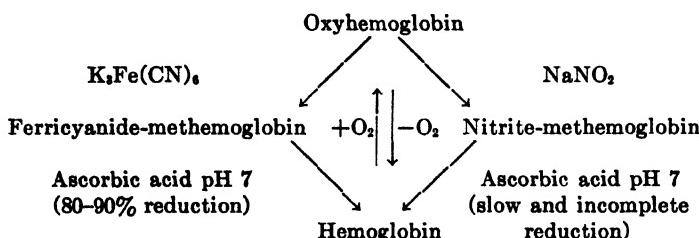
The evidence to be presented indicates that the reaction of oxyhemoglobin with potassium ferricyanide does not lead to the same product as is obtained with sodium nitrite (7–9), although spectrophotometric evidence in the visible region suggests that very similar products are obtained (9).

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* Presented in abstract before the Thirty-fifth annual meeting of the American Society of Biological Chemists at Chicago, April 19, 1941.

The oxidation-reduction potentials of the ascorbic acid system have been determined by Ball (10) and those of the hemoglobin system by Taylor and Hastings (11). With the aid of simple graphical estimation, it can be shown that the two systems at pH 7 ought to interact to an extent sufficient to reduce 83 to 95 per cent of the methemoglobin. (The oxidation-reduction titration curves found by Taylor and Hastings (11) are not symmetrical; hence the estimated range of per cent reduction.)

EXPERIMENTAL

The experimental basis for the work reported in this paper has been the determination of oxygen capacities according to Van Slyke and Neill (12), Sendroy's (13) modification which involves equilibration with air in the chamber of the manometric apparatus being employed. All pH values have been measured with a standardized Beckman pH meter. The ascorbic acid used was the Pfizer product and all other reagents were of the highest quality obtainable.

Crystalline oxyhemoglobin was prepared from fresh defibrinated horse blood[1] in the following manner, which constitutes a slight modification of the procedures of Ferry and Green (14) and of Heidelberger (15). The cells were washed four times in an ordinary centrifuge with 1 per cent sodium chloride, and then treated with $\frac{1}{4}$ of their volume of toluene. After gentle shaking, the mixture was run through the Sharples supercentrifuge to hemolyze the cells and emulsify the toluene in the system. Crystalline oxyhemoglobin precipitated in the centrifuge during the process. After several hours at 0° the resulting paste was centrifuged in an ordinary centrifuge and the toluene-stroma layer discarded. It was then treated with a minimum amount of 1 N sodium carbonate to dissolve the crystals, filtered on a Buchner funnel, and the oxyhemoglobin recrystallized by bubbling in a 4:1 mixture of oxygen and carbon dioxide at 0° until the pH reached 6.6. In this way a thin cell-free paste of crystalline oxyhemoglobin containing emulsified toluene was obtained; it was stored in the refrigerator under toluene vapor and remained stable and contaminated by only traces of methemoglobin for several months.

Ferricyanide-methemoglobin systems have been prepared by treating analyzed solutions of crystalline horse oxyhemoglobin or hemolyzed defibrinated horse blood with 1.1 molecules of $K_3Fe(CN)_6$ per hemoglobin iron atom (assuming a molecular weight of 68,000 and 4 iron atoms per molecule for hemoglobin). Nitrite-methemoglobin systems have been prepared by the addition of 1.1 molecules of $NaNO_2$ per iron atom to crystalline horse oxyhemoglobin or to washed rabbit erythrocytes. Oxygen

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[1] The author desires to express his gratitude to the Animal Pathology Laboratory at the University of Illinois for the fresh horse blood used in this work.

capacity determinations have been carried out on these methemoglobin preparations and compared with blank determinations.

The various methemoglobin systems have then been treated with freshly prepared solutions of ascorbic acid in a 1:1 mixture of phosphate buffer and isotonic sodium chloride solution (1 molecule of ascorbic acid per iron atom, a 2-fold excess) under the conditions noted in Tables I to IV, and the oxygen capacities of the resulting systems measured at various time intervals. Experiments in which the calculated amounts of ascorbic acid were used led to lower yields of oxyhemoglobin because of incomplete reduction; somewhat decreased oxygen capacities were also encountered when a 4-fold excess of ascorbic acid was used.
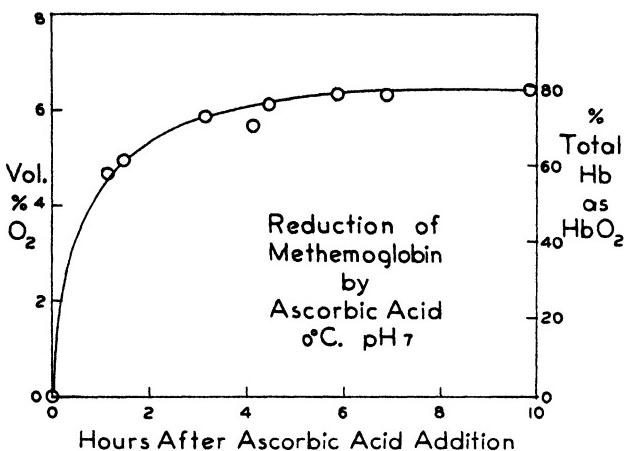
FIG. 1. Reduction of ferricyanide-methemoglobin by ascorbic acid at 0° and pH 7. O = oxygen capacities at indicated times after ascorbic acid addition.

Blank oxygen capacity determinations were carried out with each series of measurements, and the experimentally determined c corrections were subtracted in each case. The effect of the presence of ascorbic acid on the determination of oxygen capacities (d values in Tables II to IV) is worthy of mention. A slight lowering of the measured oxygen capacity was encountered in the majority of experiments when ascorbic acid was present. This effect was observed even in the blank determinations and probably indicates partial oxidation of ascorbic acid under these conditions. It was very difficult to arrive at a constant d value for a given set of conditions, since d is a function of both time and temperature and is superimposed on slow oxyhemoglobin degradation. For this reason the various d values are recorded in each case.

Fig. 1 shows the reduction of ferricyanide-methemoglobin by ascorbic acid at 0° and pH 7. The methemoglobin was prepared by adding 1.1

molecules of solid $K_3Fe(CN)_6$ per iron atom to a saline solution of oxyhemoglobin at 0°; oxidation is complete in a few minutes. After the addition of ascorbic acid, samples were withdrawn at the intervals noted and the oxygen capacities determined. On one vertical axis the volume per cent oxygen is plotted, and on the other the per cent of total hemoglobin as oxyhemoglobin; the latter values were calculated without correcting for the depressant effect of ascorbic acid on the oxygen capacity. Methemoglobin was about 60 per cent reduced and subsequently oxygenated within 2 hours and 80 per cent reduced and oxygenated within 6 hours at 0°. No attempt has been made to analyze the curve, but it seems clear that the two electromotively sluggish systems interact according to thermodynamic prediction. The coupled oxidation of ascorbic acid and hemoglobin

TABLE I

Oxygen Capacity Determinations; Effect of Ascorbic Acid on Ferricyanide-Treated Hemolyzed Defibrinated Horse Blood

System 1, hemolyzed horse blood; System 2, ferricyanide-treated hemolyzed horse blood; System 3, ferricyanide-treated hemolyzed horse blood + ascorbic acid.

System No.	O_2	Total Hb as HbO_2	Remarks
	vol. per cent	*per cent*	
1	15.35	100	
2	2.98*	19	Systems 2 and 3 kept at 0° for 24
3	13.50	88	hrs. before analysis

* Ferricyanide oxidation was not complete when 1.1 molecules of $K_3Fe(CN)_6$ were added per iron atom, which suggests that other components of the system used up part of the oxidizing agent.

described by Lemberg et al. (4) does not take place at an appreciable rate at 0°.

In Table I the effect of ascorbic acid on ferricyanide-treated hemolyzed defibrinated horse blood is illustrated. The presence of ascorbic acid brought about a marked reduction of methemoglobin in the presence of all the components of defibrinated blood. The well known inability of ferricyanide ions to penetrate the red cell necessitated hemolysis by the addition of a few mg. of saponin.

The effect of two electromotively active dyes was then investigated and the reactions carried out at room temperature. Table II illustrates the effect of methylene blue (E_0' at pH 7 = +0.011 volt) in 10^{-6} M concentration (concentration of pigment iron, approximately 0.002 M). Methylene blue (10^{-5} M) and indigodisulfonate (10^{-6} M, E_0' at pH 7 = −0.125 volt) were also tested. In each case after 1 hour at room temperature the systems were held at 0° for 4 to 12 hours. The standard potential of

indigodisulfonate is too low to allow it to exert any appreciable catalytic effect on the interaction of methemoglobin and ascorbic acid, but methylene blue is very effective in either concentration. In some cases the presence of methylene blue led after several hours to a slightly more rapid loss of oxygen capacity than was observed in its absence. At room temperature the methylene blue-catalyzed reaction reaches completion at the end of 1 hour, but secondary reactions involving hemoglobin breakdown also develop more rapidly than at 0°. The percentages of total hemoglobin as

TABLE II

Oxygen Capacity Determinations; Effect of Methylene Blue (10^{-6} M) on Reduction of Ferricyanide-Methemoglobin by Ascorbic Acid

System 1, oxyhemoglobin; System 2, oxyhemoglobin + ascorbic acid + methylene blue (10^{-6} M); System 3, ferricyanide-methemoglobin; System 4, ferricyanide-methemoglobin + ascorbic acid; System 5, ferricyanide-methemoglobin + ascorbic acid + methylene blue.

System No.	O_2	Total Hb as HbO_2	Remarks (temperature = 25°)
	vol. per cent	per cent	
1	6.80	100	
2	7.00		Analyzed immediately after addi-
3	0.01		tion of ascorbic acid
4	4.54	67	½ hr. after ascorbic acid addition
5	5.14	76	
4	5.46	80	1 hr. after ascorbic acid addition
5	6.19	91	
2	6.64		$d = 0.16$*
4	5.51	81	After 4 hrs. at 0°
5	5.88	86	

* d = loss in measured O_2 capacity due to presence of ascorbic acid.

oxyhemoglobin have been calculated without taking into account the depressant effect of ascorbic acid on the oxygen capacity.

Table III records the results of one of several experiments with nitrite-treated rabbit erythrocytes. Fresh defibrinated rabbit blood was centrifuged at 0–5° and the cells washed four times with isotonic salt solution in a refrigerated centrifuge. The washed cells were then suspended in saline solution and treated with 1.1 molecules of solid $NaNO_2$ per atom of iron for ½ hour at room temperature (29°). At the end of that time, the cells were again washed four times with equal volumes of cold saline solution in the refrigerated centrifuge. No hemolysis took place in these operations.

The data in Table III show a depressant effect of ascorbic acid on the measured oxygen capacity of intact cells. After 4 hours at 29° and 14 hours at 0°, the cells which had been treated with ascorbic acid and meth-

TABLE III

Oxygen Capacity Determinations; Experiments with Nitrite-Treated Rabbit Erythrocytes*

System 1, washed rabbit erythrocytes; System 2, washed erythrocytes + ascorbic acid + methylene blue (10^{-6} M); System 3, nitrite-treated erythrocytes; System 4, nitrite-treated erythrocytes + methylene blue (10^{-6} M); System 5, nitrite-treated erythrocytes + methylene blue (10^{-6} M) + ascorbic acid; System 6, nitrite-treated erythrocytes + methylene blue (10^{-6}M) + glucose ($10^{-1.9}$ M); System 7, nitrite-treated erythrocytes + methylene blue (10^{-6} M) + glucose ($10^{-1.9}$ M) + ascorbic acid.

System No.	O_2	Total Hb as HbO_2	Remarks (temperature = 29° ± 1°)
	vol. per cent	*per cent*	
1	9.57	100.0	
2	9.03		$d = 0.54$ after 2 hrs.
3	5.12	53.5	Analyzed 2 hrs. after nitrite treatment
4	5.81	60.7	" 2 " " methylene blue addition
5	6.35	66.3	" 2½ " " " " + ascorbic acid addition
2	9.03		$d = 0.54$ after 4 hrs.
3	5.40	56.4	Analyzed 5 hrs. after nitrite treatment
4	6.03	63.0	" 4½ " " methylene blue addition
5	7.31	76.4	" 4 " " " " + ascorbic acid addition
6	6.46	67.5	Systems prepared 6 hrs. after original nitrite treatment
7	6.68	69.8	Analyzed 1 hr. later
2	9.15		$d = 0.42$
3	6.40	66.9	All systems analyzed after 14 hrs. at 0°
4	6.62	69.2	
5	8.27	86.4	
6	9.30	97.2	After 14 hrs. at 0°
7	9.14	95.5	

* The presence of $NaNO_2$ ($10^{-1.9}$ M) or $NaNO_2$ ($10^{-1.9}$ M) + ascorbic acid ($10^{-2.2}$ M) at pH 7.2 led to no interference with blank oxygen capacity determinations.

ylene blue (System 2) had undergone no further change as far as oxygen capacity or appearance was concerned. The nitrite treatment of the cells (System 3) resulted in an initial loss of approximately one-half of the oxygen capacity. 5 hours at 29° led to only a slight recovery of oxygen

capacity; but at the end of 14 hours at 0° the nitrite-treated cells exhibited about two-thirds of their original oxygen capacity, showing a marked tendency of the intact cells to recover their oxygen capacity slowly.

The rather decided difference between Systems 4 and 5 (Table III) at the end of 4 hours at 29° and 14 hours at 0° suggests that the presence of ascorbic acid was slowly effective in bringing about the reduction of nitrite-methemoglobin. Systems 6 and 7 (Table III) serve to confirm the experiments of Warburg, Kubowitz, and Christian (16) in showing that the intact red cell which is actively metabolizing glucose has a decided ability to restore nitrite-methemoglobin to oxyhemoglobin.

TABLE IV

Oxygen Capacity Determinations; Effect of Ascorbic Acid on Ferricyanide-Methemoglobin and on Nitrite-Methemoglobin

System 1, oxyhemoglobin; System 2, oxyhemoglobin + ascorbic acid + methylene blue (10^{-6} M); System 3, ferricyanide-methemoglobin; System 4, nitrite-methemoglobin; System 5, ferricyanide-methemoglobin + ascorbic acid + methylene blue; System 6, nitrite-methemoglobin + ascorbic acid + methylene blue.

System No.	O_2	Total Hb as HbO$_2$	Remarks (temperature = 29° ± 1°)
	vol. per cent	*per cent*	
1	5.56		
2	5.79	100	$d = -0.23$ after 2 hrs.
3	−0.01		
4	−0.02		
5	5.44	94	After 2 hrs.
6	0.01	0	
2	4.73		$d = 0.83$ after 4 hrs.
5	2.91	50	After 4 hrs.
6	1.26	22	" 4½ "

The experiments on nitrite-treated erythrocytes led to a comparison of the effect of ascorbic acid on ferricyanide-methemoglobin with that on nitrite-methemoglobin (Table IV). Such a comparison shows a striking difference in the speed and extent of reduction. Ferricyanide-methemoglobin was rapidly reduced by ascorbic acid-methylene blue at 29°, but after 4 hours at that temperature secondary reactions had set in involving the breakdown of the oxyhemoglobin. The nitrite-methemoglobin, however, was untouched at the end of 2 hours at 29°, but at the end of 4½ hours about one-fifth of the oxygen capacity had been recovered. In the absence of intact erythrocytes nitrite-methemoglobin is not spontaneously converted to oxyhemoglobin.

The results above indicate that ferricyanide-methemoglobin is not identical with nitrite-methemoglobin and that nitrite-methemoglobin prepared as described in this report is not "simple" methemoglobin. It follows accordingly that visible spectrum analysis alone may not positively identify certain abnormal blood pigments.

SUMMARY

1. The reduction of methemoglobin by ascorbic acid has been studied *in vitro* at 0° and 25–30°; the reaction proceeds according to thermodynamic prediction (83 to 95 per cent reduction).

2. The presence of catalytic amounts of methylene blue greatly speeds up the reaction, while the presence of indigodisulfonate is substantially without effect.

3. Chemical evidence suggests that potassium ferricyanide and sodium nitrite do not lead to the same product (methemoglobin) when allowed to react with oxyhemoglobin.

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PROLACTIN*

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Several years ago a preliminary communication from this laboratory (1) reported the isolation of a crystalline protein with high lactogenic (prolactin) activity. The prolactin fraction used for the preparation of the crystalline product was obtained from whole beef pituitary glands by a procedure essentially that described by Lyons (2), with certain modifications. The method of Lyons yields a highly purified product which, when examined by several methods, both in this laboratory and in the laboratory of Evans in California (3, 4), gives evidence of a high degree of homogeneity. The yield of crystalline material is small, chiefly owing to denaturation of the protein which occurs during the crystallization procedure. This fact, together with subsequent observations that the original procedure described (1) is not uniformly successful, has led to a reexamination of the crystallization of prolactin. Efforts have been made to improve the crystallization method both with respect to the uniformity of results and the yield of product. A variety of procedures, successfully employed for the crystallization of other proteins, have been tried with purified, amorphous prolactin preparations; none has proved satisfactory. A new procedure has been devised which, unfortunately, suffers chiefly from one of the same drawbacks of the method originally described; namely, exceedingly low yields of the crystalline product. In the meantime, several investigators, in private communications, have reported their inability to obtain a crystalline preparation by the procedure originally described (1). On the other hand, one laboratory[1] has informed the authors that crystalline products have been obtained in low yields, although not uniformly.

It is, of course, realized that the crystallinity of a protein is not necessarily a good criterion of purity. Indeed, in certain instances in work with prolactin, crystallization has been observed to be accompanied by a decrease in lactogenic potency.[2]

The present communication describes two procedures which have been employed for obtaining a crystalline protein with marked prolactin activity. The electrophoretic behavior (5) and the ultraviolet absorption spectrum (6) of this product have already been recorded. An adequate quantity of material has now been accumulated to permit a further examination of its properties, to compare certain of these with those of highly purified prolactin preparations prepared in this and in other laboratories, and to establish definitely its protein nature.

EXPERIMENTAL AND RESULTS

Preparation of Crude Prolactin—1 kilo of whole beef pituitary glands is extracted as described by Lyons (2). The acid-acetone extract obtained is treated with 6 liters of acetone and the mixture allowed to stand in the ice box overnight. The bulk of the supernatant solution can be removed by siphoning and the remaining fluid separated from the precipitate by centrifuging.

The precipitate is extracted with four 50 ml. portions of distilled water, the extract being separated each time from a water-insoluble residue by centrifuging; the clear extracts are combined. Addition of 9 volumes of acetone to this aqueous solution precipitates the crude prolactin. After standing in the ice box overnight, the precipitate is centrifuged and washed three times with acetone. The product is dried at room temperature *in vacuo* over sulfuric acid. The range of yields from 1 kilo of glands is 1.2 to 1.9 gm.

Preparation of Purified Prolactin—The product obtained as described above contains a considerable quantity of protein which exhibits a minimal solubility at pH 6.4 to 6.8. This is the so called adrenotropic fraction (2). Separation from prolactin is effected by dissolving the crude product in 1 per cent concentration at approximately pH 8.0 with the aid of 0.1 N sodium hydroxide, and adjusting to pH 6.6 by the careful addition of 0.1 N hydrochloric acid. After removal of the precipitate which forms, the prolactin is precipitated by further addition of 0.1 N hydrochloric acid to pH 5.4. The centrifuged prolactin is then redissolved with the aid of dilute alkali as before and the hydrogen ion concentration gradually increased by the addition of 0.1 N hydrochloric acid; any precipitate which separates above pH 6.0 is removed by centrifugation. For these reprecipitations, the volume of solution used has been approximately 20 ml. The supernatant is again finally adjusted to pH 5.4 to precipitate the prolactin.

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[2] Similar observations have been made independently in the Schering Laboratories.

This procedure may be repeated four or five times to free the prolactin from contaminating protein insoluble above pH 6.0. More effective separations and better yields of purified prolactin have been obtained by the use of ammonium sulfate together with adjustment of the hydrogen ion concentration. Both the adrenotropic and prolactin fractions have a significant degree of solubility at the hydrogen ion concentrations at which they exhibit maximum flocculation. In view of this solubility, and consequent loss of prolactin in the supernatants during repeated reprecipitations carried out to effect purification, 5 ml. of saturated $(NH_4)_2SO_4$ solution have been added to every 100 ml. of the 1 per cent solution of crude prolactin, prior to the initial separation of the material insoluble at pH 6.6. 0.5 ml. of saturated $(NH_4)_2SO_4$ is used for each subsequent reprecipitation which is carried out in a volume of 20 ml. The final pH 5.4 precipitate obtained after repeated reprecipitations is dialyzed free from salt and dried by washing with acetone, as described for crude prolactin, or by the lyophile process. The range of yields of purified prolactin from each gm. of crude product is 75 to 125 mg.

Crystallization of Prolactin—Two methods have been employed for obtaining crystalline products, (a) an acetic acid-pyridine procedure which is essentially that described (7) for the crystallization of insulin, and (b) precipitation from dilute acetone solutions. For the acetic acid-pyridine procedure, 200 mg. of purified prolactin are dissolved in 2 ml. of 13 per cent (by volume) acetic acid in a 15 ml. centrifuge tube. 2 ml. of 10 per cent (by volume) pyridine, made by diluting redistilled pyridine with water, are added with stirring and the precipitate is centrifuged. The concentrations of acetic acid and pyridine employed vary somewhat from those originally used in the crystallization of insulin (7), and give somewhat clearer supernatants.[3] Following removal of the fluid by decantation, the precipitate is redissolved in 2 ml. of the acetic acid solution and precipitation carried out again by the addition of 2 ml. of 10 per cent pyridine; the precipitate is then centrifuged. This procedure is repeated ten times in all, the supernatants after each centrifugation being combined. The solution obtained in this manner is faintly turbid; it is allowed to stand in the ice box for several days. Small, hexagonal crystals will gradually deposit; the latter slowly dissolve as the solution warms to room temperature, and frequently appear under the microscope as prismatic crystals, the edges of which are somewhat rounded. A photomicrograph of a preparation which had been permitted to deposit over a 2 month period in the ice box is shown in Fig. 1.

A few remarks may be made regarding this procedure. (a) It is desir-

[3] Dr. Schwenk has informed the authors that the crystallization method employed in the Schering Laboratories is essentially the technique published by Abel and his colleagues (8), with the modification of omitting brucine from the procedure.

able to have the combined supernatants as clear as possible prior to placing these in the ice box. If any significant degree of turbidity is present, a precipitate rapidly forms at the lower temperature and this will consist of large, amorphous particles. (b) The yield of crystalline product is exceedingly small. This may be attributed largely to the fact that purified prolactin appears to suffer some loss of solubility when repeatedly redissolved in dilute acetic acid prior to the treatment with dilute pyridine. The insoluble residue remaining after ten reprecipitations amounts to 50 to 65 per cent of the weight of the amorphous prolactin taken.[4] The limited solubility of the prolactin under the conditions employed has contributed to the low yield of crystalline protein. (c) Recrystallization has not been uniformly satisfactory because of apparent denaturation of the protein, associated frequently with a decrease in biological potency.

FIG. 1. Crystalline prolactin.   × 900

Recrystallizations which have been successful have been carried out by the pyridine-acetic acid procedure as described.

In a second procedure for obtaining a crystalline product, advantage is taken of the fact that prolactin exhibits a considerable degree of solubility when precipitated from slightly alkaline solution by adjustment of the hydrogen ion concentration to the point of maximum flocculation. The protein remaining in solution may be precipitated from the supernatant fluid by the addition of acetone to a concentration of 80 per cent, followed by chilling of the solution. The microscopic appearance of the product is similar to that obtained by the pyridine-acetic acid procedure. This

[4] Bioassay of this insoluble residue indicates that a loss of 50 to 80 per cent of the physiological potency generally accompanies loss in solubility of prolactin occurring in this step of the crystallization procedure.

procedure may be carried out directly on the crude prolactin in the following manner.

500 mg. of crude prolactin are triturated in a mortar with 15 ml. of water and 0.2 ml. of 2 M sodium hydroxide. The suspension is transferred to a 50 ml. centrifuge tube with the aid of distilled water to make a total volume of 20 ml. An additional 0.2 ml. of the alkali is added; vigorous stirring facilitates solution. A small amount of undissolved protein may be evident; this is conveniently removed with the material insoluble at pH 6.6. The strongly alkaline solution is immediately treated with 0.3 ml. of 2 M hydrochloric acid which lowers the pH to the region of 8.0 to 9.0. The solution is now adjusted to pH 6.6 by the careful addition of 0.1 N hydrochloric acid; the precipitate which forms is centrifuged. The clear supernatant is then further treated with 0.1 N hydrochloric acid until the pH is 5.4 to 5.5. The precipitate is immediately centrifuged off and the slightly turbid supernatant treated with 4 volumes of acetone and placed in the ice box. On standing overnight, a crystalline sediment is deposited which under the microscope resembles the product obtained by the pyridine-acetic acid procedure.

Biological Assay—The prolactin activity of various preparations has been determined by two methods: (a) the 2 day "local" assay (9) and (b) the 4 day systemic test (10). It has been observed in the present work that the systemic method has given more satisfactory results, and this technique has been usually employed. 6 week-old pigeons of a single strain and from a single source[5] have been used. Four daily subcutaneous injections are made in the tail region, the volume of fluid of each injection being 0.5 ml. The birds are killed 24 hours after the last injection and the intensity of the response evaluated as described by Lyons (2, 10). In the assays reported in the present communication, comparisons have been made with the international standard[6] with an accepted activity of 10 I.U. per mg. In agreement with the recent publication of Lyons (10), it has been found that the activity claimed for the international standard is approximately 3 times too high. However, for purposes of comparison with other investigators, the value of 10 I.U. per mg. for the international standard has been used in evaluating the activity of unknown products. Two prolactin preparations from other laboratories have also been carefully assayed. Each of these was prepared from sheep pituitary glands; one was kindly furnished by Dr. E. Schwenk of the Schering Corporation and the other was obtained through the generosity of Dr. W. R. Lyons of the University of California. The prolactin activities of the various

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[5] The pigeons employed were all of the white Carneau strain and were obtained from the Palmetto Pigeon Plant, Sumter, South Carolina.

[6] Kindly supplied by Dr. A. S. Parkes, National Institute for Medical Research, London.

products, determined by the systemic method of assay, are presented in Table I. It will be seen that several preparations of purified prolactin, prepared in three different laboratories, have a biological activity which, within the limits of error of the assay method, may be considered identical.

Homogeneity Studies—Several of the methods generally accepted as useful for examining the homogeneity of proteins have been employed in this study of prolactin.

Electrophoresis in Tiselius Apparatus—The electrophoretic behavior of crystalline prolactin has been previously reported from this laboratory (5). Since the initial publication, the apparatus has been equipped with the schlieren scanning device of Longsworth (11). The descending boundaries (protein into protein) were scanned for the reasons advanced by Longsworth and MacInnes (12). Fig. 2 shows scanning patterns obtained in a typical experiment with a crystalline prolactin preparation (35 I.U. per mg.). The protein boundary migrated in a manner characteristic of a homogeneous preparation. Similar homogeneity was observed in an

TABLE I

Prolactin Activity of Various Preparations

	international units per mg.
Crude prolactin	10–15
Purified prolactin.....	30–35
Crystalline prolactin (pyridine-acetic acid procedure).	30–35
" " (acetone procedure)	30
Twice recrystallized prolactin (pyridine-acetic acid procedure) ..	30–35
Purified prolactin (from Dr. Schwenk)...... ....'....	30–40
" " " " Lyons)......................... .	30

electrophoresis experiment conducted at pH 3.90 (acetate buffer, ionic strength 0.05).

Solubility Studies—The crystalline prolactin employed in the solubility studies possessed a physiological activity of 30 to 35 I.U. per mg.

Three series of solubility experiments were conducted, each with a different solvent, (a) 0.12 M NaCl in 0.01 N HCl, (b) 0.33 M NaCl in 0.01 N HCl, and (c) doubly redistilled water. The pH values of these solutions were determined with the glass electrode and found to be, respectively, 2.05, 2.0, and 6.92 at 23°.

Approximately 500 mg. of prolactin were employed for each solubility study. The protein was precipitated at pH 5.5 and washed with successive portions of each solvent until two successive solubilities were the same. The precipitate was then broken up into a fine suspension and different quantities distributed among tubes filled with the solvent. The tubes were rotated for 48 hours at 23°, filtered, and the filtrates analyzed for

nitrogen by the micro-Kjeldahl method. The results of the solubility studies are shown in Fig. 3. It will be seen that the solubility is independent of the amount of the saturating solid, from the first appearance of a solid phase. No solid phase appeared before the break in each of the curves in Fig. 3; *i.e.*, before the slope became zero. Bioassays of the soluble and insoluble material did not show any physiological differences.

Sedimentation— A 2 per cent solution (pH 8.0)[7] of crystalline prolactin was examined in an analytical Beams air-driven ultracentrifuge (13) arranged for optical study in the visible region by the Toepler schlieren method, as developed for the ultracentrifuge by Philpot (14).
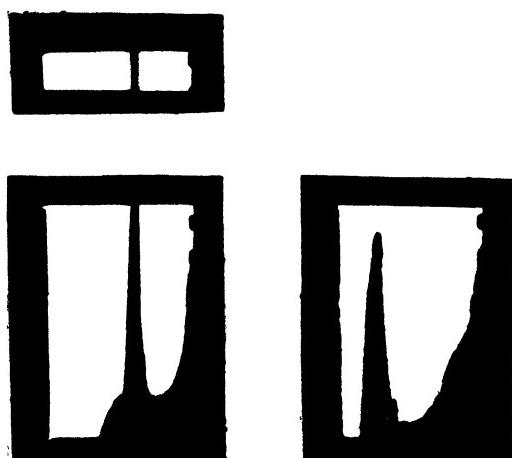
FIG. 2. Electrophoretic patterns of the descending boundary in an experiment with crystalline prolactin. Protein concentration, 2 per cent; buffer, 0.017 M phosphate (pH 8.0); ionic strength, 0.1; temperature, 6°. Photographs, from left to right, at 0 time and after 4 hours

The initial protein peak present at the start of the experiment sedimented at a uniform rate. A comparable run with highly purified, amorphous prolactin gave a similar diagram. In Fig. 4 are reproduced the photographs obtained in a run with a crystalline preparation. The experimental details are given in the legend for this figure. The average value of the sedimentation constant, S_{20}, found for prolactin in several runs was approximately 2.8 × 10^{-13} cm. sec.[-1] dynes [1]. Tentatively assuming the prolactin molecule to be spherical, the sedimentation constant obtained indicates an approximate molecular weight (15) of the order of 35,000 for this protein hormone. Recently, the molecular weight for

[7] The prolactin was suspended in water and solution completed with the aid of 0.1 N NaOH. The solution was then brought to pH 8.0 by the addition of 0.1 N HCl

prolactin has been determined by osmotic pressure measurements and a value of 26,500 reported (4).

A highly purified, amorphous prolactin preparation (35 I.U. per mg.) was sent to the laboratory of Professor J. W. Williams at the University of Wisconsin for examination in the Svedberg oil-driven ultracentrifuge.
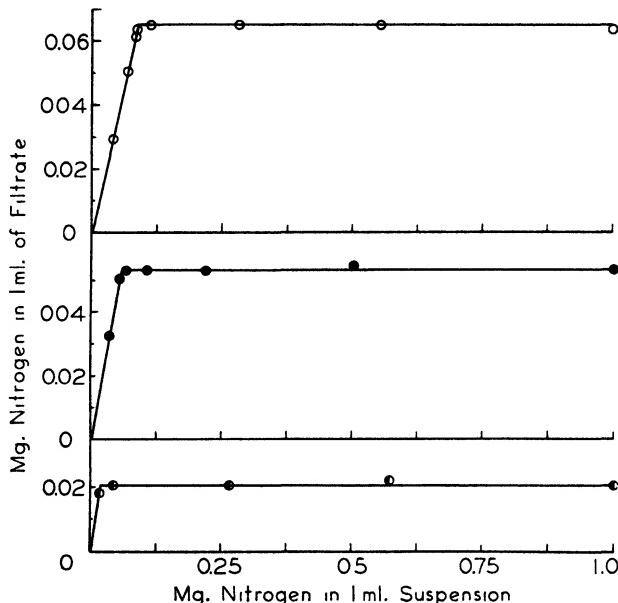
FIG. 3. Solubility curves of crystalline prolactin in various solvents. Upper curve, solvent, 0.12 M NaCl solution in 0.01 M HCl, pH 2.05; middle curve, solvent, 0.33 M NaCl solution in 0.01 M HCl, pH 2.0; lower curve, solvent, redistilled water, pH 6.92. All measurements at 23°.

FIG. 4. Sedimentation of crystalline prolactin; photographs by the Philpot schlieren method at 0, 30, 60, 90, and 120 minute intervals, reading from left to right. 45,000 R.P.M. (142,000 × g).

Several sedimentation and diffusion experiments have been completed. Professor Williams has written the following, in a private communication: "We have been able to finish a few sedimentation and diffusion experiments with your prolactin. The results we find are $S_{20} = 2.65 \times 10^{-13}$, $D_{20} = 7.5 \times 10^{-7}$, and $M = 32,000$. I am inclined to think the molecular weight value will eventually turn out to be a little larger than the figure we give.

On sedimentation and diffusion the prolactin gave curves very much like those that one would expect when working with a simple homogeneous substance."

These preliminary data obtained by Professor Williams and his colleagues will be extended when circumstances permit resumption of this work. It seems advisable to obtain as accurate physicochemical constants as possible for any highly purified protein, and the physiological interest in prolactin adds importance to data obtained in a laboratory experienced in determining these constants. Grateful acknowledgment is made to Professor Williams and his colleagues for these preliminary figures.

Isoelectric Point of Prolactin—In an early study of the electrophoresis of crystalline prolactin, Shipley, Stern, and White (5) reported an isoelectric point of approximately pH 5.6 for native prolactin. Li, Lyons, and Evans (3), from mobility studies in the Tiselius apparatus, found an isoelectric point of pH 5.70 for highly purified, amorphous prolactin. In a later communication (16), the same investigators compared the electrophoretic behavior of the lactogenic hormone prepared from sheep and beef pituitaries, and found the two preparations indistinguishable in electrophoresis experiments. An isoelectric point at pH 5.73 was reported for both products.

In view of the fact that the published values for the isoelectric point of prolactin have been based upon mobility studies in the Tiselius apparatus, it has seemed of interest to conduct a determination of the isoelectric point by the technique developed by Abramson and his colleagues (17). This method is also an electrophoretic one, based upon direct measurement of the electrical mobility of microscopically visible quartz particles coated with an adsorbed layer of protein.

For the preparation of the prolactin-coated quartz suspension, solutions of crystalline prolactin in acetate and phosphate buffers of various pH values and of constant ionic strength (0.1) were used. A small volume of quartz suspension was then added to each of the prolactin solutions and the mixtures allowed to stand approximately 15 minutes. The measurements were carried out in a modified Northrop-Kunitz cataphoresis cell (18) at 25°. The data obtained are plotted in Fig. 5. From the data, the isoelectric point of prolactin appears to be between pH 5.65 and 5.70. This finding is in good agreement with the previously published isoelectric point values of pH 5.6 (5), 5.70 (3, 4), and 5.73 (16) obtained in the Tiselius apparatus.

Elementary Analysis and Qualitative Tests—Several preparations of prolactin have been subjected to elementary microanalysis.[8] A prepara-

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[8] Elemental analyses by Mr. J. F. Alicino, who employed the Dumas method for total nitrogen. All nitrogen values have been checked by the authors, employing the micro-Kjeldahl procedure.

tion of highly purified, amorphous prolactin obtained from Dr. Schwenk has also been quantitatively examined for its elementary composition. The data obtained are presented in Table II, together with similar data obtained by Lyons.

It will be seen that the elementary composition of the prolactin preparations differs in some respects from the data reported in the preliminary communication (1), particularly with regard to the nitrogen content. The rather good analytical agreement among various preparations, taken together with the results of the bioassays, is further indication that these prolactin products are very similar to one another.[9]
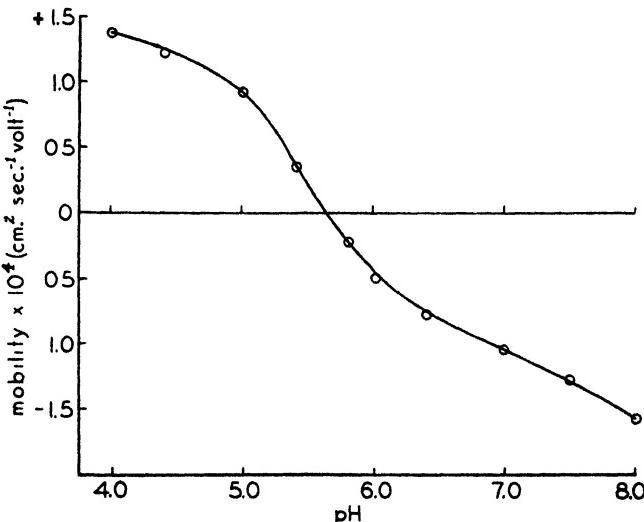
Fig. 5. The electric mobility of quartz particles covered with prolactin in acetate and phosphate buffers of constant ionic strength (0.1). The isoelectric point is between pH 5.65 and 5.70.

The crystalline preparations and the purified amorphous products give the usual protein color test (biuret, xanthoproteic, Millon's, and Hopkins-Cole). The labile sulfur test is positive. Qualitative tests for phosphorus and for carbohydrate are negative. The nitroprusside test is negative. After reduction of a 1 per cent solution of prolactin with an equal volume of 5 per cent sodium cyanide, a weak but definitely positive nitroprusside reaction is obtained provided that cyanide reduction is permitted to pro-

[9] This conclusion is further supported by the similarity of other types of data, *e.g.* electrophoretic and ultracentrifugal, which have been obtained in comparative studies with a prolactin preparation kindly supplied by Dr. Schwenk. Unpublished studies of certain physiological properties of prolactin preparations obtained from several laboratories also indicate that these products are very similar.

ceed for approximately 2 hours at room temperature. Under comparable conditions, insulin was found to give a strongly positive sulfhydryl test within 5 minutes after the addition of the cyanide.

Tyrosine, Tryptophane, and Cystine Content of Prolactin—It has been reported (19) that highly purified prolactin gave a negative Millon's test,

TABLE II

Elementary Composition of Various Prolactin Preparations

All values are calculated on an ash- and moisture-free basis and are measured in per cent.

Preparation	Carbon	Hydrogen	Nitrogen	Sulfur	Ash
Purified prolactin*.	52.04	7.01	16.84	2.05	0.59
Crystalline prolactin*	51.81	6.81	16.49	2.03	0.50
Prolactin (Schwenk)*	51.10	7.00	16.61	1.98	0.72
" (Lyons)†.	51.40	7.01	16.09	2.24	Negligible
White, Catchpole, and Long (1)* . .	51.11	6.76	14.38	1.77	"
Li, Lyons, and Evans (4)				1.79	

* Each of the analytical values for these preparations represents the average of two determinations.

† Private communication from Dr. Lyons.

TABLE III

Tyrosine, Tryptophane, and Cystine Content of Beef Prolactin

Preparation	Tyrosine	Tryptophane	Cystine
	per cent	*per cent*	*per cent*
Purified amorphous prolactin .	5.64	1.30	3.32
.	5.50	1.27	3.41
Crystalline prolactin	5.42	1.34	3.34
	5.48	1.30	
Average*	5.51	1.30	3.36
Li, Lyons, and Evans (22)	5.73	1.31	
" " " " (4)	5.7	2.5	
Fraenkel-Conrat (26).			3.0

* All values were calculated on an ash- and moisture-free basis.

indicating the absence of tyrosine. On the other hand, a positive qualitative test for this amino acid has been reported (1) with crystalline prolactin preparations and also with highly purified products (20, 21). Moreover, the presence of tyrosine in prolactin has been clearly demonstrated by quantitative analysis (4, 22) and by ultraviolet absorption spectrum studies (6).

Both crystalline and highly purified, amorphous prolactin prepared from whole beef pituitary glands have been analyzed for tyrosine and tryptophane by the micromethod of Folin and Marenzi (23). In view of the absence of carbohydrate in prolactin, no modifications in the original procedure of those authors were employed. Cystine was determined in an HCl-formic acid hydrolysate (24) by the method of Sullivan and Hess (25). The results of these analyses are shown in Table III, together with some analytical data from the literature.

It will be seen that the values obtained for the tyrosine, tryptophane, and cystine content of prolactin are in good agreement for the two preparations employed. The tyrosine value found confirms that reported for beef prolactin by Li, Lyons, and Evans (4, 22). The tryptophane content of prolactin found in the present investigation is essentially the same as that found (1.31 per cent) by Li, Lyons, and Evans (22), employing Lugg's modification (27) of the method of Folin and Ciocalteu (28). More recently, however, these same investigators have reexamined prolactin for its tryptophane content (4) and report a value considerably higher (2.5 per cent) than that initially obtained (22). The higher value is attributed to the fact that it has been obtained by a glyoxylic acid method (29) which does not require digestion of the protein, whereas the Lugg method originally employed for the determination of tryptophane involved an alkaline digestion which may result in some destruction of the tryptophane.

It is the opinion of the authors that Shaw and McFarlane (29), who have studied the glyoxylic acid method in some detail, have not established that destruction of tryptophane occurs either under exactly the conditions of hydrolysis described by Folin and his colleagues (23, 28) or in the procedure employed by Lugg (27). According to Lugg, tryptophane estimations require correction for 3 per cent loss if stannite has been included in the alkaline hydrolysis, or 6 per cent when alkali alone has been used. Brand and Kassell (30) in a careful and a thorough study of the photometric determination of tryptophane, tyrosine, diiodotyrosine, and thyroxine, based on the procedure developed by Lugg from the Folin-Ciocalteu method, have suggested that no correction factor is required for the tryptophane content of alkaline hydrolysates of proteins. In view of these observations, the considerably lower tryptophane values for prolactin reported in the present paper, as contrasted to those of Li, Lyons, and Evans, cannot be attributed to destruction of this amino acid during alkaline hydrolysis of the protein. Rather, the disagreement appears to be based on the use of methods employing incompletely as contrasted to completely hydrolyzed proteins.

The cystine value found for prolactin is slightly higher than that recently reported by Fraenkel-Conrat (26). A cystine content of 3.36 per cent accounts for approximately 45 per cent of the total sulfur of prolactin.

Effect of pH and Heat on Prolactin Activity—Prolactin has been reported (21) to be relatively thermostable when heated in a boiling water bath for 1 hour at pH 7.0 to 8.0, and less stable at other pH ranges. In view of the fact that the possible presence of other proteins in the prolactin preparations previously employed and the use of relatively concentrated protein solutions may have markedly affected the degree of hormone destruction, the heat stability of highly purified prolactin in dilute solutions of different hydrogen ion concentration has been studied.

Solutions containing 0.04 per cent of amorphous prolactin (30 I.U. per mg.) were heated in a boiling water bath for 15 and 30 minute periods at pH values over the range 1 to 13. The desired pH values were obtained with 0.01 N HCl and 0.01 N NaOH in suitable proportions. 5 ml. of the

TABLE IV

Effect of pH and Heat on Prolactin Activity

pH	Period of heating	Total dose injected for bioassay*		
		1 γ	10 γ	100 γ
	min.			
1	15	+	+	+
	30	0	+	+
3	15	+	+	+
	30	0	0	+
7	15	+	+	+
	30	0	0	+
9	15	+	+	+
	30	0	0	+
11	15	0	+	+
	30	0	0	+
13	15	0	0	+
	30	0	0	0

* + indicates positive response, active; 0 indicates negative response, inactive.

protein solution were employed for each experiment. At the end of the heating period, each sample was cooled immediately to room temperature, neutralized to approximately pH 7, made to a total volume of 10 ml., and subjected to bioassay at a total protein level of 1, 10, and 100 γ. The 2 day "local" method of Lyons and Page (9) was used in these experiments. The results are shown in Table IV.

Even though the minimum effective dose has not been determined in these bioassays, it is evident that prolactin, under the conditions employed, is quite stable to heat in 0.04 per cent solutions of pH 1 to 9 when the heating is carried out for 15 minutes in a boiling water bath. At higher pH values there is a considerable loss of biological potency. With a 30 minute heating period, there is a definite destruction of prolactin activity at all pH

values, the effect again appearing to be most marked at pH 13.   It would appear, therefore, that prolactin may be classed as a heat-labile substance; this is in harmony with the protein nature of the hormone.   The destruction observed in the present experiments might find explanation in the splitting of labile sulfur and in a significant degree of hydrolysis.

Hydrolysis of Prolactin by Acid—50 mg. of amorphous prolactin (30 I.U. per mg.) were boiled for 18 hours with 20 per cent hydrochloric acid.   After filtration and neutralization of the hydrolysate, no evidence of biological activity could be obtained, even though assays were conducted at a dose level approximately 400 times (based on the original protein concentration) that required to produce a positive response by the unhydrolyzed prolactin.

Hydrolysis of Prolactin by Pepsin—In view of the preceding experiment, hydrolysis of prolactin was carried out with proteolytic enzymes in an effort to correlate the rate of hydrolysis of the protein with alterations in biological activity.   A solution of 50 mg. of amorphous prolactin (minimum effective dose intradermally, 0.5 γ) dissolved in slightly less than 10 ml. of 0.1 N HCl and a solution of 2 mg. of granular pepsin, 1:10,000 (Wilson Laboratories), dissolved in 5 ml. of 0.1 N HCl were kept in an incubator at 37° for 15 minutes.   At the end of this time, 0.5 ml. of the pepsin solution was added to the prolactin solution, and the total volume made to 10 ml. with 0.1 N HCl.   After mixing, three 0.5 ml. samples were taken, two to be used for the determination of non-protein nitrogen and the third for prolactin assay.   Similar samples were taken at 1, 2, and 3 hours digestion time.

The aliquot taken for assay was neutralized and then made slightly alkaline (pH 7.5) with 0.1 N NaOH and allowed to stand for 15 minutes in order to stop enzyme action.   The solution was then made to a suitable volume and bioassayed.

The other two aliquots were each treated as follows: 0.5 ml. of 10 per cent trichloroacetic acid was added and, after precipitation was complete, the mixture was filtered and the precipitate washed twice with 1 ml. portions of 5 per cent trichloroacetic acid solution.   The combined filtrate and washings were digested and the total nitrogen determined by the procedure of Koch and McMeekin (31).   The final color density was determined in the Evelyn photoelectric colorimeter (32), with a 520 mμ filter.   The amount of nitrogen in the unknown sample was determined from a standard curve made with solutions containing known quantities of pure ammonium sulfate.   The increase in the non-protein nitrogen served as an index of the rate of digestion of the protein.   The data obtained, together with the bioassay results, are presented in Table V.

After 3 hours incubation time, approximately 48 per cent of the total protein had been digested by pepsin.   At the end of 2 and of 3 hours of

digestion, nearly half of the nitrogen of the protein remained in a form sufficiently complex to be precipitable by trichloroacetic acid. If this had been unaltered prolactin, there was a sufficient quantity present to give a positive prolactin response. From the complete absence of such response, even at the highest dose level tried, it may be inferred that destruction of the biological activity of prolactin by pepsin takes place relatively early in the proteolytic process, in any case before decomposition into compounds that are not precipitable by the trichloroacetic acid. In a control experiment, prolactin dissolved in 0.1 N HCl and allowed to remain at 37° for 4 hours showed no decrease in biological activity.

Hydrolysis of Prolactin by Trypsin—50 mg. of prolactin (as above) were suspended in 3 ml. of water. The protein was dissolved with the aid of

TABLE V

Rate of Digestion of Prolactin by Pepsin or Trypsin As Correlated with Alterations in Biological Activity of Hormone

	Pepsin					Trypsin			
Time	Per cent original pro-tein digested*	Bioassay, total dose level†			Time	Per cent original pro-tein digested*	Bioassay, total dose level†		
hrs.		γ	γ	γ	*hrs.*		γ	γ	γ
0	0	1‡	10‡	100‡	0	0	1‡	10‡	100‡
1	29	0.7	7‡	70‡	0.5	15	0.8‡	8‡	80‡
2	40	0.6	6	60	1.0	30	0.7‡	7‡	70‡
3	48	0.5	5	50	2.0	47	0.5	5	50
					3.5	49	0.5	5	50

* Non-precipitated by 10 per cent trichloroacetic acid solution.

† All dosages, with the exception of those at 0 time, were calculated from nitrogen analysis.

‡ Active, *i.e.* positive prolactin response. All other assays were negative at the dose levels indicated.

dilute 0.1 N NaOH and then neutralized to approximately pH 7.8. 2 ml. of Sörensen's phosphate buffer (pH 7.8) were then added and the volume made to 5 ml. with water. A trypsin preparation (Fairchild) was dissolved in the same phosphate buffer and was made up to contain 1 mg. of trypsin per ml. Both solutions were incubated at 37° for 20 minutes. 1 ml. of the trypsin solution was then added to the prolactin solution, the volume made to 10 ml. with water, and the solution mixed. Samples were taken for analysis and for bioassay at intervals for 3.5 hours. In order to stop further tryptic action, each sample on removal was immersed in a boiling water bath for 1 minute, cooled rapidly, and diluted to a convenient volume for assay. Non-protein nitrogen was determined on each of two aliquots as has been described in the pepsin experiments.

The results obtained are shown in Table V; the data are similar to those obtained with pepsin. At the end of 3½ hours digestion time, 49 per cent of the total protein was no longer precipitable by 10 per cent trichloroacetic acid solution. The bioassay results demonstrate that, although after 2 and after 3½ hours digestion time nearly half of the nitrogen of the protein remained precipitable by 10 per cent trichloroacetic acid solution, there was a complete absence of prolactin response. Thus, as in the pepsin experiment, destruction of physiological activity occurs before there has been a complete hydrolysis of the protein into fragments which are no longer precipitated by trichloroacetic acid.

The data on peptic digestion resemble those of Fisher and Scott (33) on insulin. These investigators noted that insulin activity was rapidly destroyed by pepsin and that hypoglycemic potency decreased even more rapidly than the rate of hydrolysis of the protein. The present digestion experiments likewise point to the identity of the hormone and the protein and would seem to eliminate the possibility that the biocatalytic action of prolactin can be attributed to the presence of a particular component or constituent of the protein hormone molecule.

Comment

The excellent method devised by Lyons for the extraction of prolactin from pituitary tissue led subsequently to the preparation of highly purified, amorphous preparations of this protein hormone. Indeed, detailed studies in two laboratories now appear to offer strongly suggestive evidence for the homogeneity of prolactin preparations obtained by the Lyons technique, or slight modifications thereof. The preparation of a crystalline product from highly purified, amorphous prolactin also indicates the high degree of purity of the amorphous material. This is further supported by the fact that crystallization does not significantly enhance the biological activity.

All of the data obtained in the present study, together with other published investigations, confirm the protein nature of prolactin. The physiological activity of the hormone is evidently a function of the intact protein as a whole and is not due to a particular component or constituent of the prolactin molecule. The solution of the problem of why prolactin exhibits this particular type of biological activity may of necessity have to wait for a more intimate understanding of the structure of proteins which do not contain the now recognized types of prosthetic groupings.

The preparation of a pure protein hormone of the anterior pituitary will be of considerable aid in elucidating the complex physiology of this endocrine gland, and the rate of progress in this direction will be greatly accelerated as each additional anterior pituitary secretion is made available in a purified and homogeneous state. It is naturally of great interest to

determine whether the prolactin preparations now available exhibit any other type of physiological activity which has been attributed to anterior pituitary extracts. Details of such physiological studies will be reported at a later date.

Acknowledgment is made to Miss Hilda B. Ritter for assistance in conducting the bioassays.

SUMMARY

From a highly purified amorphous preparation of beef prolactin it has been possible to prepare a crystalline protein with a lactogenic activity very similar to that of the amorphous product. The homogeneity of this protein is indicated from three types of studies, (1) electrophoresis, (2) ultracentrifugation, and (3) solubility measurements.

The following additional data are also presented for prolactin: sedimentation constant, diffusion constant, approximate molecular weight, isoelectric point, elementary analysis, tyrosine, tryptophane, and cystine content, heat stability at various pH values, and alterations in biological activity occurring when the protein is hydrolyzed with acid, pepsin, or trypsin. The data obtained in this study of prolactin confirm the protein nature of this anterior pituitary hormone.

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THE INHIBITION OF YEAST CARBOXYLASE BY SPLIT-PRODUCTS OF N,N-DIMETHYL-AMINOAZOBENZENE*

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In a recent report from this laboratory (1), evidence was presented that p-phenylenediamine and several of its methyl derivatives strongly inhibit fermentation in a washed yeast system in which diphosphopyridine nucleotide is the limiting factor for activity. The toxicity of the p-phenylenediamine derivatives varied directly with the stability of their free radicals as determined by Michaelis, Schubert, and Granick (2) and also varied according to the ease with which the compounds are oxidized by the apozymase preparation (1). The results of these experiments suggested that the inhibition of fermentation was due to the formation of an intermediary oxidation product of the aromatic p-diamine tested, probably the free radical or semiquinone. It appeared, furthermore, that the oxidation product inhibited the system by inactivating an enzyme essential to fermentation by yeast. Finally, an apparent correlation was shown to exist between the toxicity of aromatic p-diamines of the type under investigation and the carcinogenic potency (for rat livers) of the various methyl derivatives of aminoazobenzene which have been tested. The results of the experiments of Hashimoto (3) on o-aminoazotoluene and of Stevenson, Dobriner, and Rhoads (4) on N,N-dimethylaminoazobenzene indicate that both of these compounds are split *in vivo* at the azo linkage, a fact which suggests that methylated p-phenylenediamine derivatives are formed in the animal from the methylated aminoazobenzene compounds which are fed.

In view of these findings it was thought desirable to extend the study of the toxic properties of the p-phenylenediamine derivatives to yeast and tissue enzyme systems other than the diphosphopyridine nucleotide system previously studied. The experiments herein reported are con-

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cerned with the effects of these Wurster type compounds on a carboxylase system contained in alkaline washed yeast.

EXPERIMENTAL

The alkaline washed yeast (No. OF-20, Fleischmann Laboratories) carboxylase system used was that described by Ochoa and Peters (5).

In the main vessel were placed 100 mg. of alkaline washed yeast (No. OF-20), 12 γ of $MnSO_4$, 845 γ of $MgCl_2$, 15 γ of cocarboxylase, and 10 γ of thiamine. 5 mg. of sodium pyruvate, pH 6.4, were placed in the *side arm*. The volume was made up to 2 cc. with $M/15$ phosphate buffer, pH 6.4. $T = 30°$.

As has been reported for other yeasts, the addition of thiamine was found to increase the decarboxylation of pyruvic acid effected by the strain employed in these experiments. The effect on decarboxylation was most marked when low cocarboxylase concentrations were used. When high concentrations of cocarboxylase were added, thiamine had no stimulating effect on the reaction. This finding is compatible with the explanation of the thiamine effect offered by Lipton and Elvehjem (6); namely, that the reason for the stimulation of activity by thiamine is the combination of thiamine with catalytically inactive proteins which are also capable of combining with cocarboxylase. The preliminary saturation of these molecules with thiamine thus leaves all added cocarboxylase to combine with the catalytically active protein. If a large excess of cocarboxylase is added, the combination of it with the catalytically inactive molecules still leaves enough cocarboxylase free to maintain catalytic function at its maximum rate. Therefore, the addition of thiamine at high concentrations of cocarboxylase should not cause any stimulation of the rate of decarboxylation. For the yeast employed, it was found that 10 γ of thiamine gave a maximum stimulation and this amount was added to all manometers.

The Wurster type compounds were added in the form of hydrochloric acid salts in the concentrations indicated in Tables I, II, and IV.

The washed yeast carboxylase preparation used in these experiments was found to be strongly inhibited by several compounds of the series studied (Table I). As was found with the diphosphopyridine nucleotide system (1), the compounds of the Wurster series which are capable of forming stable free radicals were the most toxic and those which form very unstable free radicals were non-toxic. Thus, o-methyl-N,N-dimethyl-p-phenylenediamine is non-toxic in this system, and it can be oxidized to form a free radical which has a stability of only 5 minutes (2). However, m-methyl-N,N-dimethyl-p-phenylenediamine is markedly toxic in an equivalent concentration and, under the same conditions, is capable of forming a free radical of much greater stability; namely, 2 to 3 days.

In the diphosphopyridine nucleotide system (1) it was found that the toxicity of the compounds tested could be prevented in part if a reducing agent such as cysteine, glutathione, or ascorbic acid was added. The inhibition by the more toxic compounds of this series, however, was not greatly decreased by these reagents. It was also observed in the diphosphopyridine nucleotide system that the colored oxidation products of the more toxic Wurster salts were formed in spite of the addition of the re-

TABLE I

Inhibition of Yeast Carboxylase-Cocarboxylase System

15 γ of cocarboxylase were added to each manometer.

Compound (concentration, 5 × 10⁻⁴ M)	Per cent inhibition	Free radical stability*
N,N,N',N'-Tetramethyl-*p*-phenylenediamine............	96	Wks.
N,N,N'-Trimethyl-*p*-phenylenediamine	85	7 days
N,N-Dimethyl-*p*-phenylenediamine	81	7 "
m-Methyl-N,N-dimethyl-*p*-phenylenediamine...........	75	2 "
N,N'-Dimethyl-*p*-phenylenediamine	67	1 day
Diaminodurene.	65	2–3 days
2-Methyl-*p*-phenylenediamine	65	4–8 hrs.
p-Phenylenediamine	51	4–8 "
2,5-Dimethyl-N,N,N',N'-tetramethyl-*p*-phenylenediamine	15	1 min.
o-Methyl-N,N-dimethyl-*p*-phenylenediamine	9	5 "
Monoacetyl-*p*-phenylenediamine	0	
Diacetyl-*p*-phenylenediamine	5	
Monoacetyl-N,N-dimethyl-*p*-phenylenediamine. .	0	
N,N,N',N'-Tetramethyl-*o*-phenylenediamine	10	
p-Aminophenol	15	
N,N-Dimethyl-*p*-aminophenol	19	
Pyocyanine	10	
Methylene blue	9	
Sodium iodoacetate	5	
Alloxan	15	

* See Michaelis, Schubert, and Granick (2).

ducing agents, a fact which probably explains the failure of the reducing agents to protect the system.

In contrast to the findings with the diphosphopyridine nucleotide system in which there is much less catalysis of the oxidation of the Wurster type compounds by the washed yeast preparation the addition of cysteine and glutathione kept the Wurster type compounds in the reduced state, and prevented their inhibition of the carboxylase activity (Table II). In this system, as well as in the diphosphopyridine nucleotide system, the formation of an oxidation product of the reduced compound is essential to secure

a toxic effect. Also in support of this assumption is the fact shown for the diphosphopyridine nucleotide system that the acetylation of *p*-phenylenediamine and of the N,N-dimethyl derivative renders them non-toxic by

TABLE II

Typical Experiment Showing Effect of Addition of Cysteine on Toxicity

15 γ of cocarboxylase were present in each experiment.

Compounds added (concentration, 5 × 10⁻⁴ M)	Cysteine added	CO_2 produced in ½ hr.
	mg.	*c.mm.*
None	0	159
"	3	190
N,N-Dimethyl-*p*-phenylenediamine.	0	27
"	3	170
o-Methyl-N,N-dimethyl-*p*-phenylenediamine	0	150
"	3	206
m-Methyl-N,N-dimethyl-*p*-phenylenediamine	0	33
"	3	180
p-Phenylenediamine..... ....:.	0	78
"	3	200

TABLE III

Effect of Cysteine on Decarboxylation of Pyruvic Acid .

Experiment No.	Cocarboxylase added	Cysteine added	CO_2 produced in ½ hr.
	γ	*mg.*	*c mm.*
1	3		28
	3	0.1	32
	3	0.2	39
	3	0.3	55
	3	0.8	90
2	15		122
	15	3	237
3	15		126
	15	3	211
4	15		159
	15	3	190
5	15		138
	15	3	200
6	15		119
	15	3	228
	15	3 (Cystine)	110

stabilizing the reduced compounds and preventing their oxidation under the conditions observed. This also held for the carboxylase-cocarboxylase system (Table II).

The decarboxylation of pyruvic acid by the washed yeast cocarboxylase complex used in these experiments, like that reported by Greenberg and Rhinehart (7), was stimulated by the addition of cysteine and glutathione and was not inhibited by an equivalent amount of cystine (Table III).

It was found that in the diphosphopyridine nucleotide system the inhibition by the Wurster type compounds could be prevented by the use of higher coenzyme concentrations. The results of the experiments indicated that the inhibition was caused by a successful competition of an oxidation product of the reduced Wurster type compounds with the co-

TABLE IV

Effect of Variation of Cocarboxylase Concentration on Per Cent Inhibition

Experiment No.	Compound added (concentration, 5×10^{-4} M)	Cocarboxylase	CO_2 produced in $\frac{1}{2}$ hr.	Per cent inhibition
		γ	*c.mm.*	
I-1	None	15	202	
2	N,N-Dimethyl-p-phenylenediamine	15	39	81
3	None	400	360	
4	N,N-Dimethyl-p-phenylenediamine	400	378	0
II-1	None	3	130	
2	N,N,N′,N′-Tetramethyl-p-phenylenediamine	3	3	98
3	None	15	190	
4	N,N,N′,N′-Tetramethyl-p-phenylenediamine	15	38	80
5	None	30	352	
6	N,N,N′,N′-Tetramethyl-p-phenylenediamine	30	88	75
7	None	60	520	
8	N,N,N′,N′-Tetramethyl-p-phenylenediamine	60	260	50
9	None	1000	550	
10	N,N,N′,N′-Tetramethyl-p-phenylenediamine	1000	484	12
III-1	Diphosphopyridine nucleotide*	15	205	
2	" " * and N,N,N′,N′- tetramethyl-p-phenylenediamine	15	51	75
3	N,N,N′,N′-Tetramethyl-p-phenylenediamine	15	52	75

* The concentration was 1 mg. per 2 cc. instead of 5×10^{-4} M as elsewhere.

enzyme for an enzyme protein which requires the coenzyme to complete its catalytic function.

In the experiments with carboxylase-cocarboxylase it was also found that a variation (Table IV) in the coenzyme concentration could alter profoundly the degree of inhibition of the system. At a cocarboxylase level of 15 γ the addition of N,N-dimethyl-p-phenylenediamine (5×10^{-4} M) completely inhibited activity. The addition of 400 γ of cocarboxylase under the same conditions prevented the inhibition by the N,N-dimethyl-p-phenylenediamine. In the diphosphopyridine nucleotide

system the oxidized forms of the Wurster salts were reduced when fermentation proceeded. In this carboxylase system the colored oxidation products of the Wurster salts were not reduced when the high concentration of cocarboxylase was added. Hence, in the carboxylase system the high concentration of cocarboxylase prevented the inhibition in the presence of the oxidation products of the Wurster type salts. In the diphosphopyridine nucleotide system this point was obscured by the fact that when fermentation began the Wurster type salts were reduced.

The addition of large amounts of diphosphopyridine nucleotide to the carboxylase-cocarboxylase system did not prevent the inhibition of this system by the Wurster type compounds (Table IV). Conversely, the addition of large amounts of cocarboxylase to the fermenting system in which diphosphopyridine nucleotide was the limiting factor did not prevent the inhibition in that system. In each case the variation of the concentration of the limiting coenzyme significantly altered the magnitude of the inhibition, whereas the variation in concentration of the non-limiting or non-functioning coenzyme was without effect on the magnitude of the inhibition.

DISCUSSION

In both the diphosphopyridine nucleotide fermenting system (1) and the yeast carboxylase-cocarboxylase system several methylated aromatic p-diamines which are capable of forming stable free radicals (2) were markedly toxic. In each case the toxicity was associated with the formation of colored oxidation products from the reduced diamines. In each case the magnitude of the inhibition was found to vary inversely with the concentration of the limiting coenzyme. Thus, at low concentration of coenzyme the inhibition was nearly 100 per cent; at high concentration of the coenzyme the inhibition approached zero. Only variation in the concentration of the limiting coenzyme changed the percentage inhibition. The variation in the concentration of the non-limiting or non-functioning coenzyme in each case was without effect on the magnitude of the inhibition.

These experiments indicate that there exists in each case a competition between the oxidized Wurster salt and the limiting or functional coenzyme for the enzymes which require these coenzymes to complete their normal catalytic function. The possibility of a direct combination with an inactivation of the coenzymes is ruled out by the fact that only a variation of the concentration of the limiting coenzyme in each case has any effect on the magnitude of the inhibition. If a direct combination with the coenzymes occurred, one would expect the addition of either coenzyme in large amounts to protect each system. This was not found to be the case

(Table IV). These results indicate that the effect of the Wurster type salts is due to an inactivation of the catalytically active protein.

The fact that in equivalent concentrations the Wurster salts are not non-specific enzyme poisons (8) suggests the possibility that there is, in the sensitive enzymes, some similar molecular grouping susceptible to oxidative inactivation, which in each case acts as the active center for coenzyme activity. A second possibility is that the presence of excess coenzyme stabilizes the catalytically active protein.

On the basis of the experimental results obtained with the diphospho-pyridine nucleotide system and work reported in the literature, it was suggested that the oxidation of sulfhydryl groups might be responsible for the observed enzyme inactivation. As a part of this indirect evidence, it was cited that both alloxan and iodoacetic acid which are known to react with sulfhydryl groups also markedly inhibited fermentation. However, iodoacetic acid, as has previously been reported (9), and alloxan do not inhibit the carboxylase-cocarboxylase system (Table I). Although this does not definitely rule out a sulfhydryl group effect, it makes it a less likely possibility.

In these experiments with the carboxylase-cocarboxylase, as in the case of the diphosphopyridine nucleotide fermenting system, there again is an apparent correlation between the toxicity gradient of the Wurster type compounds and the carcinogenic potency of the corresponding aminoazo-benzene derivatives ((1) Table VIII).

SUMMARY

1. The addition of thiamine and cysteine was found to stimulate the rate of decarboxylation of pyruvic acid by the yeast carboxylase preparation used.

2. Several compounds of the Wurster type series were found to inhibit the decarboxylation of pyruvic acid in this system. The toxicity gradient paralleled, as in the diphosphopyridine nucleotide yeast fermenting system, the ease of formation and the stability of the intermediary oxidation products (free radicals).

3. The addition of reducing agents such as cysteine and glutathione to this system prevented its inhibition by the Wurster type compounds.

4. The experiments indicate that the inhibition is a competitive one. The results of the experiments reported added to the results of those performed with the diphosphopyridine nucleotide system (1) indicate that the coenzymes are not inactivated and that the toxic effect is due to in-activation of the catalytically active proteins.

5. As was the case in the diphosphopyridine nucleotide system, the toxicity gradient of the Wurster type salts parallels the carcinogenic

potency (rat livers) of the corresponding methyl derivatives of aminoazo-
benzene from which they are probably formed *in vivo*.

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LIPIDS OF THE FASTING MOUSE

II. THE FAT TO WATER RELATION IN THE LIVER AND THE FRACTIONATION OF THE LIVER PHOSPHOLIPIDS*

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It has been shown recently (1) that the young adult mouse is of especial interest as an experimental animal in fat metabolism studies, because, under the physiological stimulus of fasting, all the available stored fat is mobilized (and metabolized) with great rapidity. The liver of such a fasting mouse shows a marked increase in neutral fat and the direct dependence of this fatty change on the amount of available body fat has been established (1). The liver total phospholipids remain constant in percentage despite dramatic changes in other lipid constituents.

In the present report, data on the liver phospholipids have been obtained in greater detail; the lecithin to cephalin ratios have been determined and these compounds further fractionated into their α and β forms. From these findings it has become evident that the constancy in total phospholipid is a result of compensatory changes in the specific types of phospholipid present. Quantitative data are also presented on liver total lipid and its iodine number, liver water, and liver chloride. In their bearing on the general problem of lipid metabolism, the current findings on the changes in the liver lipids of fasting mice offer several new lines of thought both as to the nature and as to the function of liver phospholipids.

EXPERIMENTAL

3 month-old, male, albino mice previously maintained on a diet of oats and Purina dog chow were used. The fasting procedure was as previously reported (1) except that the temperature was maintained at 25°. The mice were killed by decapitation and blood collected for plasma chloride determinations (2). The livers were removed quickly, one lobe (the same one in each case) was taken for water and chloride (2) determinations, and the remainder weighed, placed in 95 per cent ethyl alcohol, and analyzed by standard lipid methods for total lipid (3), phospholipid (4), and iodine

number (5). The lecithin percentage of the total phospholipid was determined by Taylor's (6) modification of the methods of Beattie (7) and of Kirk (8) for choline analysis. Since the sphingomyelin content of liver is very small, the lecithin percentage was calculated directly from the choline content. The fractionation of the phospholipids into α and β forms was carried out according to the procedures of Suzuki, Yokoyama, and Nishimoto (9–11). The statistical procedure given by Fisher (12) was applied when data for certain group averages were compared to other group averages. Differences were considered significant when the probability of chance occurrence was not greater than once in 100 trials; i.e., $P \leq 0.01$.

Results

Mortality—The mortality rate was much lower than in a previous comparable study (1). Under the conditions of the current experiment no mice died until the 4th day of fasting, when a 22 per cent mortality was observed. The principal difference in technique of fasting was the maintenance of a higher and more constant room temperature, *viz.* 25°, whereas in the previous study the temperature varied from 18–23°. Such temperature control may well explain the mortality difference; Chevillard, Hamon, and Mayer (13) have demonstrated a marked increase in mortality with a decrease in environmental temperature.

Body Weight Loss—The average initial body weights were 20 to 21 gm. On fasting, the mice lost weight in an almost linear fashion; the day to day average changes were significant in each case. A 31 per cent loss was observed by the 4th day of fasting. This value checks exactly the previous observation (1).

Liver Weight Loss—The liver weight, normally 6 per cent of the body weight, decreased in a roughly linear fashion to 3 per cent of the initial body weight by the 4th day of fasting. This decrease in liver weight represents a loss of 48 per cent of the original weight.

DISCUSSION

The ability of the young adult mouse to mobilize depot fat has already been established (1); in the first 48 hours of fasting, all the available depot fat (1.6 gm.) is used and about one-sixth of the body cholesterol (8 mg.) is mobilized. From the data in Tables I and II it may be seen that during this period the liver total lipid increased markedly (on the average 2- to 3-fold); thirteen mice had 10 to 15 per cent, three mice had 15 to 20 per cent, and one mouse had 23 per cent total lipid (moist liver weight). The increase in liver lipid is accounted for by a 4-fold increase in the neutral fat fraction.

In the previous report, the liver total lipid was found to increase during the 1st day of fasting and thereafter to decrease. In the present study an increased lipid content was observed on the 1st and 2nd days (Fig. 1). The maintenance of high liver lipid values for the 2nd day probably is a result of the higher environmental temperatures maintained in this experiment; the mice lost body heat less rapidly and the metabolic demands were not quite so acute. This supposition is also supported by the marked reduction in mortality rate.

The mobilization of the body lipids probably proceeds by a non-selective utilization of the available fat (1). The iodine number of the *carcass* lipids has been shown to increase from about 87 to 105; a small increase occurs on the 1st day of fasting, and a large increase on the 2nd day coincident with the removal of the last of the mobilizable fat. This increase can be accounted for if lipids of average iodine number 80 had been removed non-selectively.

In the present study, the average iodine number of the *liver* total fatty acids falls from about 125 to 115 during the 1st day of fasting (Fig. 1) and remains at the latter level until the 4th day, when a marked further decrease to about 90 occurs (Tables I and II). In each case, the decrease has a high probability of statistical significance ($P < 0.01$); this is true for the decrease on the 4th day despite the more marked variation in iodine numbers as the fasting progressed.

From the iodine number changes, the state of inanition may be described in two phases of liver reaction. During both phases, the liver loses weight, in the first phase despite the added neutral fat. In this phase, the newly deposited fat produces the decrease in average iodine number which would be expected and which has been frequently described when depot fat moves into the liver. The second phase differs in that (a) on the 3rd day there is a loss of neutral fat with no change in iodine number of the residual lipid and (b) on the 4th day there is a further loss of neutral fat with a marked decrease in the iodine number. Thus, in the second phase, there is at first no evidence of a change in the saturation of the residual lipids, but later, when only phospholipid remains, there is a marked decrease in iodine number as if the phospholipid fatty acids were exchanged for more saturated acids. In line with the mass of evidence already accumulated on the structural lipids, particularly phospholipids, the removal of metabolic lipids from the carcass would be expected to leave lipids which "contain characteristically the more highly unsaturated acids" (14) and the transport of the metabolic lipids to the liver would lead to a gradual decrease in iodine number of the liver lipids (Sinclair (15)).

The concept of two kinds of processes in the succession of changes in the fasting liver is also borne out by studies of the water and chloride contents.

TABLE I

Data on Liver Lipids and Liver and Blood Chlorides of Normal and Fasted Mice

Mouse No.	Initial weight	Carcass weight*	Liver weight	Liver per gm. mouse†	Sample weight	Total lipid	Total fatty acid I No.	Phospholipid	Lecithin	Phospholipid fractionation					Liver H₂O	Chloride	
										Total phospholipid sample	α-Lecithin	β-Lecithin	α-Cephalin	β-Cephalin		Liver	Plasma
	gm.	*gm*	*gm.*	*mg.*	*gm.*	*mg.*		*mg.*	*per cent phospholipid*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*m.eq. per kg.*	*m.eq. per l.*
Normal mice																	
125	21	18.50	1.226	62	1.096	58	122	34	64	9.5	9.9	2.8	9.9	7.9	71.0	31.9	118
126	19	17.14	1.149	63	1.030	80	114	33	62	9.4	8.0	6.3	8.5	10.8	68.4	32.8	122
127	20	17.77	1.044	56	0.921	59	123	33	60	9.4	12.2	7.6	5.8	9.4	69.5	32.6	118
128	20	17.74	1.285	68	1.103	60	138	39	63	10.8	12.6	8.9	6.3	9.6	69.9	29.5	114
129	21	18.94	1.286	64	1.146	80	127	39	59	11.1	9.2	7.4	6.4	7.1	68.0	29.9	113
130	18	16.21	1.079	63	0.966	83	114	32	60	9.2					68.3	33.3	116
131	20	18.13	1.185	62	1.060	65	125	36	60						70.1	31.4	116
132	18	15.43	1.473	87	1.317	74	120	49	58						70.8	31.7	114
133	19	16.89	1.064	59	0.956	54	123	35	57						70.1	32.1	115
134	20	18.09	1.401	72	1.262	78	115	43	58	12.4	14.6	10.1	7.0	11.1	69.0	28.8	117
135	21	19.18	1.110	55	1.006	46	131	37	58	10.6	12.9	7.0	6.3	9.4	70.8	31.3	118
136	22	20.37	1.212	56	1.093	52	129	41	56	11.8	11.6	3.9	7.0	9.1	69.3	27.7	115
137	20	18.44	0.928	48	0.817	47	127	33	61	9.2	12.1	6.7	3.8	10.2	69.7	33.4	116
138	20	17.57	1.259	67	1.141	75	122	41	58	11.8	13.9	5.5	9.7	13.0	69.4	35.9	
139	21	19.33	1.255	61	1.120	58	131	44	57	12.5	16.8	10.2	6.0	7.0	69.9	34.1	111
140	20	17.91	1.453	75	1.296	63	128	44	56	12.5	12.0	7.0	8.8	7.0	71.3	34.9	117
141	24	20.04	2.592	115	0.994	120	131	92	55						68.8	29.1	114
142	21	19.04	1.176	58	1.073	53	128	43	58	12.5	13.6	10.2	7.4	12.7	69.7	30.3	115
143	22	19.70	1.066	51	0.950	51	132	37	55	10.4	12.1	7.9	5.6	11.3	68.2	29.5	118
144	22	19.57	1.071	52	0.953	54	125	38	58						69.2	31.2	115
Average	20.4	18.30	1.265	65	1.065	66	125	41	58	11.0	12.2	7.2	7.0	9.7	69.6	31.6	116
s	1.5	1.28	0.34	13	0.13	18	6	13	3	1.3	2.2	2.3	1.7	2.0	1.0	2.2	2.3

Mice fasted 24 hrs.																	
145	20	16.87	1.034	58	0.904	120	111	33	52	9.2	6.1	9.4	3.7	5.2	63.6	28.4	114
146	22	17.73	1.044	56	0.960	58	120	34	45	9.9	9.5	8.6	10.4	7.2	69.7	31.6	116
147	20	15.62	1.386	82	1.218	65	120	40	56	11.3	9.3	9.8	13.1	6.0	70.1	32.1	107
148	21	16.87	1.010	57	0.881	74	117	34	48	9.4	11.2	9.8	9.3	4.8	66.2	33.5	120
149	22	17.93	1.004	53	0.927	86	112	33	55	9.9	9.7	10.2	9.9	5.3	60.9	27.6	110
150	21	17.12	1.075	63	0.936	103	106	32	59	9.0	6.4	10.3	9.2	4.6	61.0	26.3	108
151	22	17.71	1.142	61	1.013	126	106	34	57	9.6	7.7	4 2	8.0	12.6	58.7	26.5	112
152	20	15.94	1.078	63	0.937	74	123	34	58	9.4	6.3	10.8	6.3	7.3	68.8	29.7	112
153	20	15.68	1.047	62	0.944	129	110	31	59	8.9	10.8	11.8	5.5	4.6	63.2	24.7	115
154	22	17.66	1.096	58	0.975	86	110	33	53	9.9	8.3	11.8	5.8	4.8	62.6	25.2	116
155	21	15.72	1.079	64	0.955	96	117	34	61	9.7	6.5	8.3	6.0	3.7	66.0	24.8	111
156	20	15.26	1.113	68	0.984	88	112	32	56	9.1	6.2	9.3	6.2	3.7	62.7	25.0	104
157	20	17.17	1.072	59	0.956	104	117	35	59	10.0	7.6	9.6	5.1	7.0	64.4	26.0	107
158	20	16 02	1.035	61	0.920	115	113	32	64	9.2	5.9	11.9	8.2	6.6	65.1	25.3	102
159	21	15 67	1.000	60	0.869	95	113	30	62	8.5	6.7	13.2	5.6	2.8	66.2	25.3	108
160	20	16.28	1.053	61	0.934	162	112	33	63						61.8	23.9	111
161	20	16 59	1.001	57	0.878	145	108	31	61	8.7	8.7	9.6	8.5	2.9	61.0	25.1	115
162	21	17.64	0.978	52	0.814	165	104	28	61	7.6	6.8	8.0	7.8	5 3	60.3	21.7	
163	20	14.94	1.014	64	0.889	115	115	34	56	9.7	8.5	10.4	9.2	4.6	64.0	25.6	108
164	21	15 49	1.257	75	1.130	127	113	38	57	10.8	8.8	12.8	10.1	4.4	66.4	28.3	109
Average	20.7	16 50	1.076	62	0.951	107	113	33	57	9.5	8.0	10.0	7.8	5.4	64.1	26.8	111
s	0.9	0.95	0.10	6	0.09	30	5	3	5	0.8	1.6	2.0	2.3	2.2	3.2	3.0	1.4

* Carcass not including liver weight.
† Mg. liver per gm. mouse = mg. liver per gm. carcass + liver weight.

TABLE II

Data on Liver Lipids, and Liver and Blood Chlorides of Fasted Mice

Time fasted	Mouse No.	Initial weight	Carcass weight*	Liver weight	Liver per gm. mouse†	Sample weight	Total lipid	Total fatty acid I No.	Phospholipid	Lecithin	Phospholipid fractionation‡					Liver H₂O	Chloride Liver	Chloride Plasma
											Total phospholipid sample	α-Lecithin	β-Lecithin	α-Cephalin	β-Cephalin			
days		*gm.*	*gm.*	*gm.*	*mg.*	*gm.*	*mg.*		*mg.*	*per cent phospholipid*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*m.eq. per kg.*	*m.eq. per l.*
2	165	21	15.78	1.006	60	0.912	213	97	27	54	7.6	4.6	8.3	5.3	3.7	59.3	20.7	106
	166	24	17.07	0.926	51	0.816	28	96	26	56						71.9	23.9	111
	167	20	14.22	0.876	58	0.768	144	109	27	56	7.6	6.6	6.3	6.3	5.8	59.8	18.8	109
	168	20	15.09	0.812	51	0.701	31	128	29	56						71.0	27.7	115
	169	21	14.57	1.032	66	0.927	199	110	32	53	9.3	6.7	9.8	6.9	5.5	57.4	19.5	108
	170	20	14.66	0.879	56	0.778	49	134	30	58	8.3	6.8	9.2	7.4	5.4	68.8	29.0	112
	171	21	15.36	0.996	61	0.879	118	111	28	59						65.1	28.3	113
	172	20	14.54	0.781	51	0.700	69	113	26	56	8.1	4.5	9.0	9.0	2.7	64.7	27.5	117
	173	22	15.40	0.917	56	0.814	84	113	30	58						63.6	22.5	114
	174	23	17.49	1.090	59	0.967	130	115	37	57	6.8	7.1	13.5	10.7	5.7	62.5	24.4	114
	175	20	13.97	0.806	55	0.741	66	116	28	56	7.8	7.5	8.8	6.7	4.7	64.6	27.1	116
	176	20	14.33	0.875	58	0.761	31	131	27	60						70.6	24.0	112
	177	22	15.98	1.064	62	0.930	162	114	33	62	9.3	7.0	9.7	9.4	4.2	59.0	20.9	111
	178	20	13.59	1.202	81	1.080	314	110	31	53	8.8	10.9	8.1	3.8	8.1	49.9	18.7	116
	179	21	14.74	0.985	63	0.859	64	118	32	58	8.9	7.5	9.0	4.5	9.3	67.6	26.1	113
	180	21	14.99	0.975	61	0.840	92	114	33	58	9.0	8.1	9.2	8.8	7.7	65.7	28.8	116
	181	22	15.14	0.943	59	0.872	100	100	28	64	10.5	9.4	10.5	7.7	8.2	62.9	29.3	109
	182	22	14.58	0.921	60	0.782	91	104	29	58						62.6	22.2	111
	183	20	14.74	1.142	72	1.034	154	104	36	57	8.3	8.2	8.6	6.7	6.2	61.8	22.6	112
	184	22	15.49	0.908	55	0.785	62	109	32	54						66.5	22.7	105
Average		21.1	15.08	0.957	60	0.847	110	112	30	57	8.5	7.3	9.2	7.2	5.9	63.8	24.2	112
s		1.2	0.96	0.11	7	0.10	72	10	3	3	1.0	1.7	1.6	2.0	1.6	5.2	3.6	3.4

3

No.																	
185	22	13.99	1.046	70	0.991	64	126	34	63	9.5	7.0	11.0	11.1	3.3	68.2	33.2	112
186	22	13.43	0.643	46	0.578	19	122	18	50	8.4	6.2	8.6	13.2	2.5	70.7	24.7	107
187	20	12.44	0.869	65	0.778	49	133	29	55	7.6	4.0	10.0	7.5	2.7	68.1	23.1	
188	21	13.24	0.840	60	0.733	34	147	27	56	9.0	4.3	11.2	10.7	3.8	68.4	29.7	113
189	22	14.62	0.904	58	0.796	62	121	33	52	8.0	6.7	8.2	7.2	5.3	65.9	26.8	
190	22	13.75	0.778	54	0.713	33	141	29	56						68.0	28.1	109
191	21	13.64	0.814	56	0.721	39	128	27	62						68.0	31.2	112
193	20	13.22	0.807	58	0.736	36	121	28	54						67.6	30.8	101
192	20	13.17	0.780	56	0.703	17	74			6.8	3.4	6.8	6.3	2.2	73.5	34.0	
194	20	12.52	0.698	53	0.623	21	148	22	49	8.7	5.6	7.1	7.1	4.8	70.2	30.1	114
195	20	14.61	0.640	42	0.568	15	86	23	60	8.0	5.9	7.2	7.6	2.9	71.1	31.1	108
196	21	13.11	0.860	62	0.766	71	110	24	62						64.0	23.3	113
197	20	13.61	0.781	54	0.692	61	112	30	55						65.7	26.3	111
198	22	14.73	0.962	61	0.871	37	125	21	53	6.4	5.3	8.8	6.0	2.6	69.3	28.9	111
199	22	14.39	0.739	49	0.670	46	118	28	53						65.2	28.1	107
202	21	13.56	0.882	61	0.790	99	115	12	49						61.7	24.9	114
200	20	12.90	0.524	39	0.474	16	94								66.9	53.8	
201	22	14.09	0.794	53	0.720	20	80	25	55						68.4	32.4	131
203	21	13.86	0.741	51	0.653	33	98								66.9	29.7	111
204	20	13.97	0.950	64	0.843	66	103	23	61						65.5	30.7	117
Average..	21.0	13.65	0.803	56	0.721	42	115	25	56	8.0	5.4	8.8	8.5	3.4	67.7	30.0	112
s.........	0.9	0.65	0.12	8	0.12	23	21	5	5	1.0	1.2	1.7	2.6	1.1	2.7	6.4	6.3

Table II—*Concluded*

Time fasted	Mouse No.	Initial weight	Carcass weight*	Liver weight	Liver per gm. mouse†	Sample weight	Total lipid	Total fatty acid I No.	Phospholipid	Lecithin	Total phospholipid sample	α-Lecithin	β-Lecithin	α-Cephalin	β-Cephalin	Liver H₂O	Chloride Liver	Chloride Plasma
days		*gm.*	*gm.*	*gm.*	*mg.*	*gm.*	*mg.*		*mg.*	*per cent phospholipid*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*m.eq. per kg.*	*m.eq. per l.*
4	205	22	13.82	0.736	51	0.664	26	115	22	49						69.5	31.1	108
	206	22	13.17	0.802	57	0.706	28	134	25	43					2.7	68.5	27.7	111
	207	21	13.84	0.668	46	0.593	28	102	20	44	6.7	4.0	8.4	9.3		70.0	27.3	
	208	20	12.60	0.676	51	0.610	20	116	19	46						71.7	45.6	115
	209	22	10.94	0.589	51	0.521	7	78	20	49						65.9	30.7	113
	210	20	11.84	0.658	53	0.598	35	105	21	50						68.4	33.1	113
	212	20	12.28	0.812	62	0.741	25	112	24	57					2.0	70.6	33.0	
	211	21	12.55	0.600	46	0.522	14	76	16	61	6.6	3.1	7.8	8.2				
	213	21	11.91	0.519	42	0.450	15.	93	11	62						71.9	34.3	
	214	20	13.62	0.547	39	0.479	13	69	13	56						73.1	36.9	111
	215	20	12.77	0.738	55	0.658	29	89	21	62						67.0	28.4	115
	216	22	12.40	0.684	52	0.592	18	71	15	61						71.7	31.8	107
	217	20	13.36	0.634	45	0.557	16	88	18	60						72.0	22.9	110
	218	21	12.49	0.661	50	0.569	24	97	20	55						68.4	31.3	114
	219	22	13.02	0.575	42	0.500	14	64	13	63						71.4	28.4	107
	220	20	13.09	0.576	42	0.493	14	76	16	55						69.8	24.9	115
	221	20	12.33	0.439	35	0.367	10	71	10							72.2	34.7	119
	222	20	12.29	0.686	53	0.591	25	85	21	62						68.3	26.9	108
	223	21	13.04	0.666	49	0.573	16	70	17	61						71.4	28.6	111
	224	20	12.64	0.698	52	0.594	33	110	25	53						67.5	28.9	
Average..		20.8	12.70	0.648	49	0.569	20	91	18	55	6.6	3.6	8.1	8.8	2.4	70.0	30.9	112
s.........		0.9	0.71	0.09	7	0.09	8	20	4	7						2.0	5.0	3.6

* Carcass not including liver weight.

† Mg. liver per gm. mouse = mg. liver per gm. carcass + liver weight.

‡ In pooled samples, the average mg. of total liver phospholipid were used in calculating the phospholipid fractions in percentages.

The liver water decreases about 7 per cent of its normal value in 1 day, remains nearly constant at that level for the 2nd day, and then rises regularly to an approximately normal value by the 4th day (Fig. 2). The entrance of neutral fat on the 1st and 2nd days will account quantitatively for the decrease in water content. These decreases were clearly significant statistically ($P < 0.01$) in contrast to the slight decrease observed by Fenn (16) in the water content in livers of fasted rats. The liver chloride
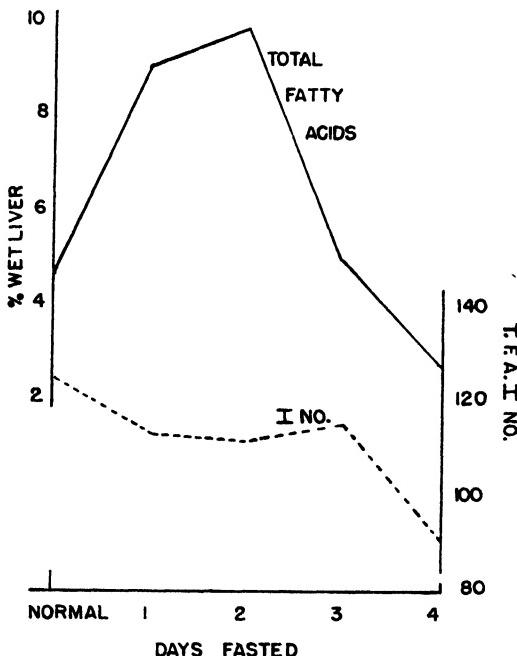
FIG. 1. The effect of fasting on the liver total fatty acids and their iodine number. Total fatty acids increase during 48 hours of fasting; thereafter fall to about half of the normal value. The iodine number of the total fatty acids falls gradually during the first 3 days and markedly on the 4th day.

presents a somewhat similar picture; it decreases about 25 per cent of its normal value in 1 day, in the 2nd day drops an additional 25 per cent, but thereafter rises to a normal value by the 4th day (Fig. 2).

When a liver becomes infiltrated with fat, the additional fat is deposited intracellularly. Consequently, in each unit of liver volume, there should be an increase of cell space at the expense of extracellular space. That this decrease in extracellular space occurs can be shown by calculations from the data for the first 2 days of fasting. The extracellular space is measured by the relation between the liver chloride and the plasma chloride content.

The latter was found to decrease from the normal value of about 115 milliequivalents per liter to 110 to 112 on the 1st day, which level was thereafter maintained (Fig. 2). Calculations showed that the "chloride phase" of the liver, normally about 26 per cent of the liver volume, decreased on the 1st and 2nd days of fasting to 23 and 21 per cent, respec-
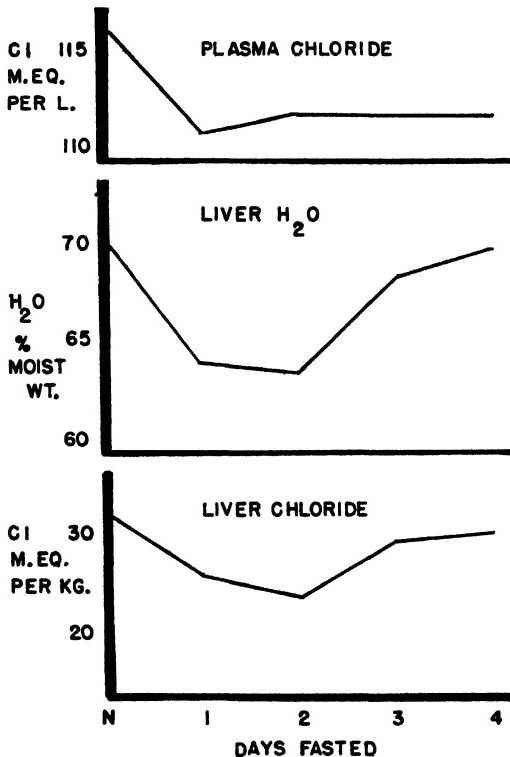
FIG. 2. The effect of fasting on liver water content and on the plasma and liver chloride levels. Plasma chloride, liver water, and liver chloride contents decrease on beginning fasting. Plasma chloride values remain at the lower level during the fasting period. In contrast, the liver water and chloride contents increase to nearly normal values on the 3rd and 4th day.

tively, and then increased on the 3rd and 4th days to practically normal values; viz., 25 and 26 per cent, respectively. Truax (17) has shown that the chloride phase is approximately identical with extracellular space; thus, the effect of the dilution (by fat) of the liver substance is clearly indicated by the decreases in chloride phase in the first 48 hour period. The normal values for extracellular volume on the 3rd and 4th days are additional evidence that a second phase of liver reaction has been insti-

tuted, a phase in which the liver function is not complicated by the presence of abnormal amounts of fat.

Our findings are in line with the hypothesis that when fat is deposited in liver cells the consequent cell enlargement results in a diminished extracellular space per unit liver volume. Fenn (16) found an increase in the extracellular space in rat liver after a 48 hour fast. However, he also showed that no increase over the normal liver fat percentage occurred in this fasting period, a typical behavior according to Best and Ridout (18). The increase in chloride space under these circumstances can be related to a depletion of glycogen and, in the absence of fat deposition, a shrinking of the cells. The decrease in extracellular space in fasted mouse liver must therefore represent a resultant of two opposing processes, of which one tends to increase extracellular space by glycogen depletion and the other, more important in this case, tends to decrease extracellular space by intracellular deposition of fat.

The water content of the fat-free liver cells (expressed as gm. of water per 100 gm. of fat-free cells) averages 63.2 for normal liver and does not differ significantly for the groups fasted 1 and 2 days. On the 3rd and 4th days, the averages show a significant decrease to 61 or 62 gm. of water per 100 gm. of fat-free cells. These data also fit into the hypothesis given above, since (a) no change in cell water would be expected if the fat deposition diluted cell material without otherwise greatly changing the cell water distribution, and since (b) a decrease in cell water content would accompany the removal of fat if glycogen depletion had occurred. The latter phase may involve a reduction in cell size without a great alteration in cell structural components.

The question of whether "excess" neutral fat is a simple diluent of liver substance or whether concomitant changes in water occur is not a new one. Fenn and Haege (19) have calculated that in cat liver the lipid constituents are only diluents and that no water accompanies fat deposition. However, they find in recalculating the data of Kaplan and Chaikoff (20) that a small but measurable amount of water is "associated with the deposition of lipid" in dog liver. Frequently, in such tests, some severe experimental procedure is instituted; e.g., the administration of various poisons to produce massive increases in the liver fat content. In the current investigation, the liver fat in mice has been caused to vary from 1 to 23 per cent (moist liver weight) by an essentially physiological treatment and therefore the water to fat relation has special interest. All the evidence which can be drawn from these data indicates that, in fasting mice, neutral fat enters the liver without disturbing the water relationships in the cells other than by dilution.

The phospholipid concentration in the liver during fasting has been

described as "remarkably constant" (1); thus, although the average values
previously obtained varied from 3.67 to 3.04 per cent (moist liver weight)
in the several groups, there was no significant difference between the aver-
ages.   In the present data, the average phospholipid percentages for the
normal groups and those fasted 1, 2, and 3 days are even more constant
(Fig. 3), probably as a result of the greater uniformity of initial body
weight.   The average phospholipid percentages for these four groups fall
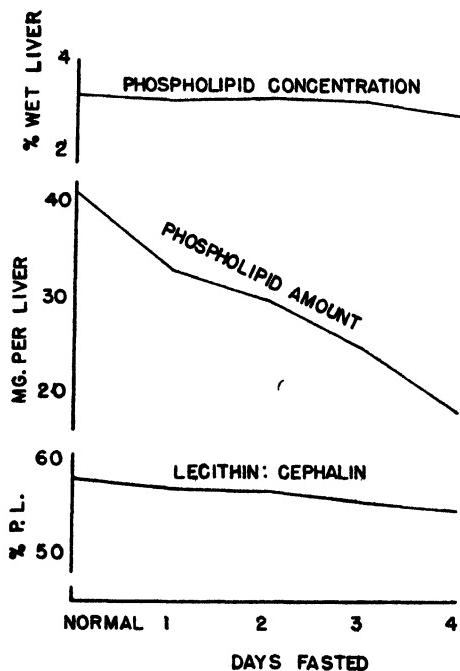
FIG. 3. The effect of fasting on the liver phospholipid concentration, absolute
amount, and lecithin to cephalin ratio.   The phospholipid concentration is constant,
although the absolute amount decreases by half.   The lecithin to cephalin ratio re-
mains unchanged during 4 days of fasting; the ordinate shows the lecithin percentage
of total phospholipid.

within the range of 3.16 ± 0.05.   However, for the 4th day the average
is 2.81 per cent, which is significantly lower than the normal.   Even this
fact can be taken as evidence of the greater uniformity in the mice, since
a difference of 0.4 per cent is significant in this series, whereas a difference
of 0.6 per cent was not in the previous series.   In general, the liver phos-
pholipid concentration shows a remarkably limited range of variation
during fasting.

In contrast, the amount of phospholipid per liver decreases regularly

and significantly from day to day in fasting (Fig. 3). This decrease is nearly linear and of such magnitude that the phospholipid is reduced to 56 per cent of the normal amount by the 4th day of fasting. Since the liver weight also decreases linearly in this period to about 50 per cent of its original value, the phospholipid concentration remains essentially constant.

The lecithin to cephalin ratio is also constant (1.38:1.22) during the 4 day fast. Although the average lecithin percentages of total phospholipid decrease from 58 to 55 (Fig. 3), none of the changes (day to day or over-all) is statistically significant.

These three findings, (a) the constancy of phospholipid concentration, (b) the loss of half the phospholipid originally present, and (c) the constancy of the lecithin to cephalin ratio, describe the over-all changes in the phospholipids. However, no suggestion is given of the striking changes which became evident on determining the amounts of α- and β-lecithin and cephalin in these livers.

A recent report by Yoshinaga (21) on the fractionation of lecithin and cephalin has indicated that in a number of normal tissues (rabbit, dog) α-lecithin occurs in larger amounts than β-lecithin (roughly 80:20) and that α-cephalin occurs in lesser amounts than β-cephalin (roughly 15:85).[1] Among others, these tissues include heart, lung, kidney, spleen, muscle, and liver. Specifically, the relative amounts of these compounds in the *liver* of normal dogs and rabbits has been reported in percentage of lecithin

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[1] The reliability of the methods used in the separation of the α and β isomers has not been studied by independent investigators. Some caution should be used in accepting the quantitative data, especially in the light of the findings of Bailly (22) and Verkade and associates (23) who demonstrated a tendency for equilibria to be set up between the isomers. However, in the data of Tables I and II may be found evidence for reasonable faith in the qualitative changes. (a) Although the amounts of total phospholipid analyzed vary from 6 to 12 mg. and there is a consistent decrease in the average total phospholipid sample with increasing duration of fasting, the standard deviations for the data on the amount of each isomer are remarkably constant; viz., 1.6 to 2.6. This constancy indicates that the over-all procedure was duplicated from sample to sample. Furthermore, a series of fractionations was run on aliquots from a large pooled sample of mouse liver. Aliquots of total phospholipid were chosen at 15, 12, 8, and 4 mg. Compared to the largest aliquot, the recoveries were within about 15 per cent of that calculated for each of the smaller samples. No trend of consistent loss or gain in any fraction was observed except for β-cephalin, where a tendency for a loss of material amounting to about 0.5 mg. was found. (b) All samples were treated alike, yet a marked trend was found; e.g., the consistent decrease in mg. of α-lecithin, which scarcely could be attributed to isomerism induced by the procedure. (c) Finally, whether the separation procedure gives in each fraction a pure isomer cannot be rigorously demonstrated at present. However, the amounts given in the various fractions are reproducible quantities. This whole study has recently been repeated and extended with a general confirmation of the findings of Tables I and II in respect to α and β isomer changes during fasting.

and of cephalin, respectively, as follows: α-lecithin, dog, 82 to 97, rabbit, 71 to 103; β-lecithin, dog, 11 to 14, rabbit, 6 to 32; α-cephalin, dog, 11 to 27, rabbit, 6 to 24; β-cephalin, dog, 75 to 100, rabbit, 70 to 99. Rae (24), using an enzymatic hydrolysis, has shown that ox liver lecithin has about equal proportions of the α and β isomers. No other information is available; considerable variation seems to exist, but, in general, α-lecithin and β-cephalin are found in preponderance, together constituting a major portion of the total phospholipid.

In normal mouse liver, the distribution of the isomers presents a similar picture but the magnitudes of the ratios are different. The relative percentages are as follows: α-lecithin, 63, β-lecithin, 37, α-cephalin, 42, β-cephalin, 58. Expressed as percentages of the moist liver weight, the values are as follows: α-lecithin, 1.0, β-lecithin, 0.6, α-cephalin, 0.6, β-cephalin, 0.8. Although α-lecithin and β-cephalin predominate in normal mouse liver, their isomers are present in relatively large amounts.

With fasting, these phospholipids show prompt and significant changes in percentages, both absolute and relative. α-Lecithin and β-cephalin decrease in a nearly linear fashion to values of 0.5 and 0.3 per cent, respectively (moist liver weight), by the 4th day of fasting.[2] The decreases in α-lecithin and β-cephalin are to about 70 per cent of their original values; the day to day decreases are statistically significant in each case. Simultaneously, the isomeric forms, β-lecithin and α-cephalin, increase to 1.1 and 1.2 per cent, respectively. The β-lecithin increases to 40 per cent above its original level on the 1st day of fasting and thereafter falls regularly to about 10 per cent above normal by the 4th day. Except for the 4th day, these increases are all significantly above normal. Although α-cephalin does not increase significantly on the first 2 days, significant increases are observed thereafter, reaching 25 per cent above normal by the 4th day. Thus, on a percentage basis, during fasting, the α-lecithin and β-cephalin decline sharply, and at the same time their isomeric forms increase to a level numerically equal to the original α-lecithin and β-cephalin levels.

A clearer insight into what is happening to the phospholipids may be gained by examining the amounts in mg. of each isomer per liver in the normal and fasted groups (Fig. 4). There are about 12 mg. of α-lecithin in a normal mouse liver; by the 4th day of fasting, only 3.6 mg. remain. Similarly β-cephalin decreases from about 10 mg. to 2.4 in the same period. In contrast, the amounts of β-lecithin and α-cephalin vary surprisingly

[2] Data are available for only two mice on this day. No statistical comparisons of these data with those for other fasted groups have been attempted. However, the data are included, since they apparently fall in line with the observations on other days.

little.   Considering these data (Fig. 4), the three findings presented above concerning the behavior of the *total* phospholipid take on new significance. (a) The phospholipid concentration is constant because as the liver loses half its weight the α-lecithin and β-cephalin almost disappear, but their isomers remain constant or even increase slightly, so that roughly half of the original phospholipid remains.   (b) The decrease in amount of phospholipid is traced to the loss in α-lecithin and β-cephalin only.   (c) The constancy in the lecithin to cephalin ratio is accounted for by the simul-
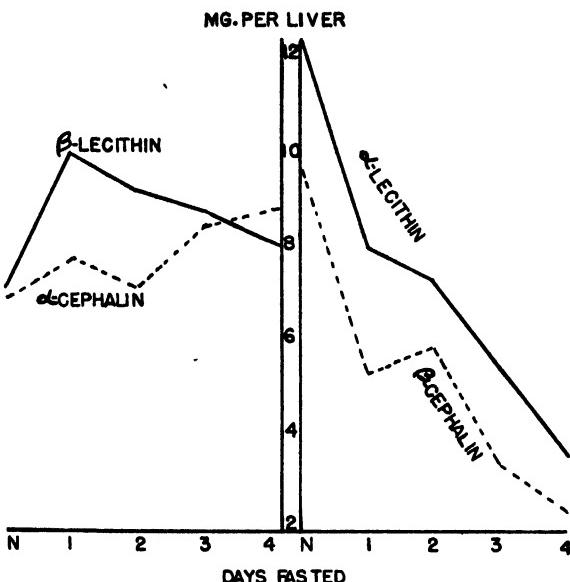
FIG. 4. The effect of fasting on the amounts of the phospholipid isomers in mouse livers.   In mg. per entire liver, α-lecithin and β-cephalin decrease in a nearly linear fashion during fasting.   By contrast, β-lecithin and α-cephalin tend to remain constant in amount.   The *N* on the abscissa refers to normal mice.

taneous and parallel losses of the α form of lecithin and the β form of cephalin.   Calculated on the basis of mg. of the isomers recovered in the phospholipid fractionation of samples obtained on the 1st and 4th days of fasting, the over-all lecithin to cephalin ratio is constant at 53 per cent lecithin.   These values check those from the choline determinations within the experimental error.

The prominent differences between the changes in the various phospholipid isomers are unexpected and difficult to explain.   Are these two, β-lecithin and α-cephalin, the true "élément constant" of Mayer and Schaeffer?   If they are built into the cell structure, how are the iodine

number changes described above brought about? Do the structural phospholipids of the liver exchange their fatty acids during the metabolism of fasting in such a way that the phospholipid molecule continually keeps in equilibrium with the fatty acids supplied in the liver without losing its location in the cell structure? Such a behavior is assigned the liver proteins by Schoenheimer, Ratner, and Rittenberg (25) in which amino acid residues may be removed and replaced apparently without involving the degradation and synthesis of entire molecules. The transformation of proteins by the exchange of free groups has been suggested by Schmidt, Allen, and Tarver (26). Can this doctrine of the "replacement of parts" also be applied to a turnover in the fatty acids and the phosphate portions of the structural phospholipids?

The α-lecithin and β-cephalin may perhaps be described as metabolic phospholipids (Sinclair (15), Artom, Sarzana, and Segrè (27)), because they tend to disappear in fasting and presumably owe their normal existence to the normal lipid metabolism of the liver. They may also be considered as "labile" phospholipids which are drawn on in the emergency of inanition. A change from α- to β-phospholipid and vice versa, although it seems unlikely, is not beyond the range of possibility. Enzymes capable of hydrolyzing α- and β-phospholipids have been isolated from liver (28–30). If such transformations take place during fasting, it would be even more interesting from the physiological point of view.

The view previously expressed (1) that, "In its lipid distribution the liver of the fasted mouse is like the normal liver" now requires drastic revision. There was no reason to suspect (lacking analyses of phospholipid isomers) that the "remarkable constancy" in the phospholipid percentages was based on a complicated balance of losses and gains of certain components. The fasted liver is not at all like the normal liver, either in its lipid distribution or in its water relationships. However, this abnormality may be turned to useful purpose. It is possible that, on resumption of feeding, the metabolic processes in the fasted liver may differ from those occurring normally. Perhaps if such metabolic differences do appear, the α-lecithin and β-cephalin will reveal in their absence part of the function they normally perform. Experiments based on this knowledge of liver lipid distribution seem to offer many possibilities of gaining new light on the problem of lipid metabolism.

SUMMARY

1. During the first 2 days of fasting, the 3 month-old, male, albino mouse deposits a large excess of neutral fat in the liver; the total lipid doubles or trebles in amount. The total fatty acid iodine number decreases immediately with fasting from 125 to 112, at which level it remains until the 4th day, when a further decrease to 91 is observed.

2. The plasma chlorides decrease on fasting from 116 milliequivalents per liter to 112, which level is maintained. The liver water and chloride contents decrease markedly during the first 2 days of fasting, and thereafter increase to about their original values. Calculations indicate that the neutral fat which enters the liver during the first 2 days of fasting does so without disturbing the liver water relationships other than by diluting the liver substance. There is evidence that during the 3rd and 4th days of fasting some other process obtains, which involves a shift in the extracellular to cellular water ratio.

3. The weight of phospholipid decreases by half during the 4 days of fasting. The liver also decreases by half in weight, so that the phospholipid concentration remains constant over all. The lecithin to cephalin ratio is not altered appreciably.

4. 80 per cent of the α-lecithin and β-cephalin originally present disappears in 4 days of fasting. In contrast, the amounts of β-lecithin and α-cephalin remain constant or increase slightly. These changes combined will account for the seeming constancy of phospholipid concentration and of lecithin to cephalin ratio.

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A POLYSACCHARIDE PRODUCED BY THE CROWN-GALL ORGANISM*

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In previous studies of the nutrition and carbon metabolism of the crown-gall organism, *Phytomonas tumefaciens* (Smith and Town) Bergey *et al.*, it was found that a considerable quantity of unidentified material was produced when the organism was grown in synthetic medium (1, 2). Because of the pathogenicity of this organism its metabolic products are of special interest. The unidentified material has therefore been investigated with the result that a polysaccharide has been isolated in apparently pure form. This polysaccharide is distinct from the gum produced by this organism and studied by Conner *et al.* (3). It is a major metabolic product and is excreted into the medium. Under the conditions employed for growth it accounted for 15 to 20 per cent of the sugar metabolized and amounted to 40 or 50 per cent of the previously unidentified material. This product is also of interest because of its relatively low molecular weight and its high degree of solubility in water. The preparation and chemical studies of this polysaccharide are presented below.

EXPERIMENTAL

Total reducing sugar was determined by the method of Stiles, Peterson, and Fred (4). For iodometric estimation of aldoses the procedure of Hinton and Macara (5) was used. Carbon analyses on bacterial cultures were made by the wet combustion method of Heck (6). Nitrogen was determined by a micromethod described by Johnson (7). Optical rotations were measured at room temperature with a Schmidt and Haensch polarimeter, No. 52b, with monochromatic light.

Culture and Medium—The culture, *Phytomonas tumefaciens* A-6, was the progeny of a single cell (8). Its pathogenicity was checked at intervals by inoculation of tomato plants and by the subsequent development of galls. The synthetic medium contained sucrose 2, urea 0.05, potassium

phosphate buffer, pH 7, 0.1, ammonium sulfate 0.1, sodium chloride 0.02, calcium chloride monohydrate 0.01, magnesium sulfate heptahydrate 0.02 per cent, 5 γ of ferric iron as ferric alum, and 2 γ of zinc as zinc sulfate per ml. The iron and the phosphate buffer were sterilized separately and added to the medium just before inoculation.

Although manganese is important for the growth of this organism, the medium was not supplemented with this element because it promotes bacterial cell formation at the expense of the metabolic products investigated (2). All cultures consisted of 200 ml. of liquid medium in 1 liter Erlenmeyer flasks and were aerated by mechanical shaking. Each flask was fitted with a 4 inch extension neck and covered with a double layer of six-ply, oil-treated air filter tissue. Incubation was at 26° ± 0.5° for 5 days. At the end of this period approximately 70 to 80 per cent of the sucrose was metabolized.

Isolation of Polysaccharide—For isolation of the polysaccharide, a group of 5 day-old cultures was combined (4 or 5 liters) and bacterial cells were removed by means of a steam-driven Sharples supercentrifuge. Barium acetate was added in slight excess to remove the phosphate and sulfate ions. The solution was concentrated to about one-twentieth the original volume, and an equal volume of 95 per cent ethanol was added to precipitate the gum studied by Conner *et al.* (3). The precipitate was removed by centrifuging, and the supernatant solution was poured with constant stirring into either 95 per cent or absolute ethanol sufficient to make the final concentration of alcohol at least 90 per cent. The precipitate consisted almost entirely of polysaccharide.

It was purified by repeatedly dissolving in water, adding an equal volume of alcohol, centrifuging, and pouring the clear solution into enough alcohol to make a final concentration of at least 90 per cent. After the second reprecipitation any barium present was removed by adding the necessary amount of dilute sulfuric acid. After one more reprecipitation any sulfuric acid remaining was removed by treatment with barium carbonate. Treatment with norit was often necessary before a pure white product was obtained. In the later reprecipitations, best results were obtained by dissolving the material in water to make a 10 to 20 per cent solution, adding alcohol dropwise with stirring until the solution became turbid, and then pouring into an excess of alcohol with constant stirring. The highest yield of polysaccharide was about 3 gm. per liter of culture.

Purity of Polysaccharide—Although attempts to crystallize the material and its acetate were unsuccessful, there is good evidence that it was essentially homogeneous and pure. The nitrogen content of most preparations was very low (around 0.04 per cent), indicating a negligible amount

of protein contamination. Preparations from several different lots of cultures showed the same specific rotation. Moreover, this property was constant through several reprecipitations with different preparations. Fractional precipitation of the material with alcohol and water gave preparations with specific rotations between $-9°$ and $-10°$.

The acetylated compound also appeared homogeneous. Attempts to separate it into different fractions were unsuccessful. 5.25 gm. of acetylated material were dissolved in chloroform and fractionally precipitated by the gradual addition of petroleum ether. Properties of the four fractions obtained are given in Table I.

From the determination of the diffusion constant (described later) one of the scale displacement diagrams was analyzed by the method of

TABLE I

Results of Fractional Precipitation of Acetylated Polysaccharide

The material was acetylated with acetic anhydride and dry pyridine at 35° for two 2 day periods.

Fraction No.	Weight	Specific rotation	Acetyl value*	Nitrogen content	Specific rotation after deacetylation
	gm.	degrees	per cent	per cent	degrees
1	0.85	+58.5	41.3	0.03	−12.7
2	1.70	+58.3	41.8	0.03	−12.6
3	1.95	+58.9	41.8	0.03	−13.1
4	0.75	+55.8	41.8	0.02	−13.2

* This value was determined by treating the acetate with 0.5 N potassium hydroxide in 50 per cent methanol for 12 to 24 hours and titrating the excess alkali.

moments; the points lay either upon or very close to the ideal distribution curve. This may be considered good evidence that the material was homogeneous.

Properties and Composition—The best preparation was a white amorphous material which could be ground to a very light powder. The material was very hygroscopic, highly soluble in water, quite soluble in pyridine, but insoluble in alcohol. Aqueous solutions could be concentrated to clear syrups, and the dry material would dissolve in water almost as readily as would sucrose. The material gave no color reaction with iodine. It was non-reducing to Fehling's solution, but iodometric titration indicated one free carbonyl group in 30 to 40 anhydroglucose units. This may be an indication of some impurity.

Because of the low molecular weight of this polysaccharide it would be difficult to obtain ash-free preparations by electrodialysis. The ash con-

tent of most preparations was about 1.5 per cent, nitrogen about 0.04 per cent.

> *Elementary Analysis*[1]—On ash-free basis
> $(C_6H_{10}O_5)_n$. Calculated, C 44.8, H 6.2; found, C 45.2, H 6.7
> *Specific Rotation*—$[\alpha]_D^{25} = -10°$ (in water, $c = 2$)

Hydrolysis and Identification of Sugar Units[2]—Hydrolysis of the material in 1 N HCl at 98° was complete in 80 to 90 minutes and gave yields of 93 to 95 per cent reducing sugar calculated as glucose. Iodometric titrations always indicated that the reducing sugar was 100 per cent aldose. During hydrolysis, the specific rotation of the solution slowly changed from $-10°$ to between $+52°$ and $+53°$, the final rotation being calculated on the basis of the amount of sugar found by analysis. Qualitative tests for fructose, uronic acids, and pentoses were negative. During the hydrolysis samples were removed for sugar analysis at various intervals, and, when the amount of reducing sugar liberated was plotted against time, a smooth curve was obtained.

As further evidence that glucose was the only sugar present, glucobenzimidazole was prepared by the method of Moore and Link (9) in sufficient yield to warrant this conclusion. 1 gm. of reducing sugar (calculated as glucose) from the hydrolyzed polysaccharide yielded 1.12 gm. of potassium gluconate, and 0.5 gm. of the potassium gluconate yielded 0.41 gm. of crude benzimidazole (72 per cent yield). This was recrystallized to give 0.24 gm. of pure material (42 per cent yield), m.p. 213°, which showed no depression of the melting point when mixed with an authentic sample of *d*-glucobenzimidazole.

In view of the yield of glucobenzimidazole, the iodometric titration results, and the specific rotation of the hydrolyzed material, it is highly improbable that any sugar other than glucose was present. The rate of hydrolysis and the smooth hydrolysis curve indicate the pyranoside inner ring structure (10). The shift in rotation during hydrolysis (from $-10°$ to $+52°$) suggests that β linkages predominate.

Acetylation—1 gm. of polysaccharide was dissolved in a mixture of 20 ml. of dry pyridine and 20 ml. of acetic anhydride and acetylated under different conditions of time and temperature. At the end of the reaction period the acetyl derivative was precipitated by pouring the mixture into ice water. After several hours the precipitate was collected in a centrifuge

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[1] Elementary analyses were made by Mr. William R. Sullivan in the laboratory of Professor Karl Paul Link.

[2] Much of the characterization work was done in the laboratory of Professor Karl Paul Link of the Department of Biochemistry. To him and to Dr. Robert J. Dimler we express thanks for their interest and suggestions.

tube, resuspended in water, let stand for 1 or 2 hours, centrifuged again, and dried in a vacuum desiccator. It was purified by dissolving in chloroform and precipitating with petroleum ether. Two acetylations of the same sample at 30° over a 4 day period gave a product with acetyl content of 41.8 per cent, $[\alpha]_D^{25} = +58°$ (in chloroform, $c = 1$). With two successive acetylations at 80° for 24 hours each, the product had an acetyl value of 44 per cent, $[\alpha]_D^{25} = +57.5°$ (in chloroform, $c = 1$). The theoretical acetyl value for anhydroglucose triacetate is 44.8 per cent. That there was no particular change in the polysaccharide as a result of acetylation and deacetylation was shown by the rotation of the regenerated product. After the acetyl value was determined, the sample was evaporated under reduced pressure to remove alcohol and then made to volume with water, and the optical rotation read. The specific rotation was $-12°$ to $-13°$ as compared to $-10°$ of the original polysaccharide.

Elementary Analysis of Acetate—
    $(C_6H_7O_5 (CH_3CO)_3)_n$. Calculated. C 50.0, H 5.56
                    Found.   " 51.5, " 5.8; acetyl value 43.7

Molecular Weight—For the calculation of the molecular weight the diffusion constant (D_{20}), the sedimentation velocity constant (s_{20}), and the apparent partial specific volume (V) were determined.[3] The apparent partial specific volume in water was 0.609 for a 1 per cent solution and 0.617 for a 1.73 per cent solution. These values agree well with figures given for other polysaccharides.

The sedimentation velocity constant was determined by the scale method in the standard Svedberg oil-driven, high speed ultracentrifuge (11) at a speed of 64,000 R.P.M., with the polysaccharide dissolved in a 1 per cent sodium chloride solution. The value of s_{20} corrected to pure water solution was 1.17×10^{-13} for a 1 per cent and 1.27×10^{-13} cm. per second per unit field for a 0.5 per cent solution of the polysaccharide.

For the diffusion constant the blurring of a boundary of a 1 per cent solution of the polysaccharide in 1 per cent aqueous sodium chloride against the salt solution was followed with time by the refractive index method. The diffusion constant was determined by the method of areas and maximum height of the scale displacement diagrams. The average value of several determinations gave $D_{20} = 2.11 \times 10^{-6}$ cm.[2] per second. When the figures $s_{20} = 1.2 \times 10^{-13}$, $D_{20} = 2.11 \times 10^{-6}$, and $V = 0.61$ are substituted in the equation $M = RTs/D(1 - V\rho)$, a calculation of the molecular weight gives 3600 ± 200 ($T = 293°$) (11). This value corresponds to a molecule which contains 22 anhydroglucose units.

SUMMARY

The isolation and characterization of a polysaccharide-produced by the crown-gall organism are presented. The polysaccharide appeared to be homogeneous as judged by analysis of diffusion data and by constant rotation through reprecipitation and fractionation of both the original material ($[\alpha]_D^{25} = -9°$ to $-10°$, in water, $c = 2$) and its acetylated product ($[\alpha]_D^{25} = +56°$ to $+58.5°$, in chloroform, $c = 1$). Hydrolysis of the polysaccharide gave only $d(+)$-glucose as indicated by specific rotation, iodometric titration, and yield of glucobenzimidazole. A shift in rotation during hydrolysis indicated a predominance of β linkages, while the rate of hydrolysis and the shape of the hydrolysis curve suggested that the inner ring structures were exclusively pyranoside. The molecular weight calculated from sedimentation velocity and diffusion constants was 3600 ± 200, which corresponds to about 22 anhydroglucose units per molecule.

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FURTHER OBSERVATIONS CONCERNING THE PREPARATION AND PROPERTIES OF CATALASE FROM DIFFERENT SOURCES .

I. CRYSTALLINE LAMB LIVER CATALASE

II. PREPARATION OF CRYSTALLINE BEEF LIVER CATALASE BY USE OF ACETONE OR ALCOHOL

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I. *Crystalline Lamb Liver Catalase*

The crystallization of lamb liver catalase has been mentioned previously (1) without a description of the preparation. Lamb liver catalase is more difficult to obtain in crystalline condition than beef liver catalase, but is more easily prepared than crystalline horse liver catalase. The method of preparation is as follows: Mix 300 gm. portions of finely ground lamb liver with 400 cc. portions of 40 per cent dioxane, and allow the resulting suspension to stand in the ice box overnight. Filter, discard the precipitate, and add 15.6 cc. of dioxane to every 100 cc. of filtrate. Allow the material to stand in the ice box for about 12 hours and filter. Discard the precipitate, add 10.3 cc. of dioxane to every 100 cc. of filtrate, and let the material stand in the ice box for another 12 hours. Now filter and allow the precipitate, which consists of crude catalase, to drain fairly dry. All of the above filtrations must take place in the ice box. Schleicher and Schüll No. 595 filter paper has been used.

The precipitate of crude catalase is scraped off the opened filters and is transferred to enough distilled water to make a thin cream. This cream is immediately filtered in the ice box and, if the filtrate is muddy, owing to the presence of glycogen, a small amount of saliva is added. After hydrolysis of the glycogen a small amount of precipitate which may have formed is filtered or centrifuged off and discarded.[1]

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[1] In order to avoid excess dioxane, which later on may result in the formation of insoluble enzyme crystals, it is of advantage at this point to adjust the pH of the enzyme solution to 7.4 with disodium phosphate, and dialyze against several changes of distilled water for 3 to 6 hours, or until spheroids just begin to form. Then filter the material and precipitate the catalase by rapidly adding an equal volume of saturated ammonium sulfate solution with stirring. Let stand in the ice box for an hour, centrifuge down the catalase, and dissolve by adding water and several drops of 10 per cent phosphate buffer, pH 7.4. The solution should be slightly less than saturated with respect to protein. If any precipitate remains, filter or centrifuge, and crystallize the catalase as described in the next paragraph.

Now enough dioxane is added to the clear, dark brown solution of catalase to make the solution 3.0 per cent with respect to dioxane, and the catalase is caused to crystallize by the cautious addition of saturated ammonium sulfate solution. Usually one-quarter of a volume of saturated ammonium sulfate can be added before crystallization starts, but when this volume is nearly reached the ammonium sulfate should be added very slowly, and, if a slight permanent clouding should occur, the addition must be discontinued at once. The catalase is then placed in the ice box and allowed to remain there for 1 hour. If any amorphous precipitate has formed, it is centrifuged down and discarded. The addition of ammonium sulfate is continued, a few drops at a time, until a good crop of crystals has formed, giving a very silky appearance on stirring. For this first crystallization, usually 2 or 3 days should be taken in order to obtain crystals large enough
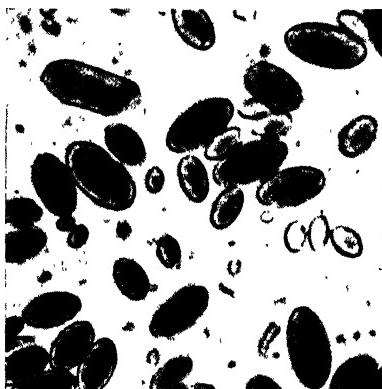
FIG. 1. Lamb liver catalase by dialysis, × 600

to centrifuge down in a reasonable length of time. These crystals consist of very fine needles. If more dioxane than the amount specified is employed, the catalase will become denatured.

To recrystallize, the once crystallized catalase is dissolved in a small amount of water and a few drops of 0.5 M phosphate buffer of pH 7.4 are added if necessary to complete the solution. Some amorphous precipitate which remains is centrifuged down and is discarded. Then either the above process of addition of dioxane and saturated ammonium sulfate is repeated, or the material is buffered to pH 7.4 and is dialyzed until nearly all of the catalase has precipitated. This latter procedure yields rounded prisms which are shown in Fig. 1. That these are really crystals is indicated (a) by the presence of birefringence, (b) by the fairly constant axial ratio and uniform internal appearance, (c) by the formation of angles when the crystals are dissolved slowly, (d) by the fact that the material has been

crystallized as needles previously, and (e) by the fact that similar rounded prisms are often obtained upon dialysis of beef liver catalase, usually together with forms intermediate between these and the sharp prisms. In the case of beef catalase, the solubility behavior of the rounded prisms appears the same as that of the prisms with sharp angles. It is likely that rounded forms of both beef and lamb catalase are caused by the presence of impurity, but the high *Kat. f.* (30,000) indicates that such impurity must be present in small amount.

If the pH is 5.5 or below at the start of dialysis, lamb catalase precipitates as fine, amorphous material.

It is seen from the above discussion that lamb liver catalase can be obtained as needles and rounded prisms which are similar to the corresponding crystal forms obtained from beef liver catalase, but so far the thin plate form of crystal (2) has not been obtained. Lamb liver catalase on dialysis shows greater solubility than that of beef catalase, but less than that of horse liver catalase.

The *Kat. f.* values of several samples of once recrystallized lamb liver catalase were found to be about 30,000. The iron content was between 0.1 and 0.2 per cent, indicating the presence of a small amount of the iron-containing impurity, ferritin. No doubt this could be removed by careful ammonium sulfate precipitation in the manner employed for horse liver catalase (3). Both hematin and the bile pigment-iron complex are present in lamb catalase, as shown by treating lamb liver catalase with acetone and hydrochloric acid as already described (2).

II. Preparation of Crystalline Beef Liver Catalase by Use of Acetone or Alcohol

In the first publication on crystalline beef liver catalase (4) we reported that we were unable to obtain the crystalline material using acetone or alcohol in place of dioxane. A study of conditions necessary for crystallization has made it possible to employ acetone or alcohol and to obtain crystalline catalase of the same activity shown by catalase prepared with dioxane. The new method in which acetone is used is of importance because it is relatively cheap and is free from the hazards of the poisonous solvent dioxane. Moreover, preparation of crystalline catalase with acetone or alcohol shows that the solvent dioxane, heretofore untried in preparing crystalline proteins, has no influence peculiar to itself in producing the crystallizable enzyme.

Using acetone, one extracts 300 gm. portions of well ground beef liver with 400 cc. portions of 35 per cent acetone, allows the material to stand in the ice box for about 12 hours, and then filters. Next, 11.6 cc. of acetone are added to each 100 cc. of filtrate and the material is allowed to stand in the ice box for 6 to 12 hours. The precipitate is then filtered off and dis-

carded.   Finally, one adds 15.9 cc. of acetone to every 100 cc. of this second filtrate and allows the material to remain for another 12 hours in the ice box. Then the precipitated catalase is filtered off in the cold and is allowed to drain fairly well. ` All of the above filtrations must take place in the ice box. The precipitate of crude catalase is scraped up with a spatula and is dissolved in sufficient water to give a thin cream.   One now filters and adds saliva if necessary to hydrolyze glycogen.   If not enough water has been used, the catalase may partly crystallize on the filter paper, resulting in marked loss of enzyme.   The catalase solution is now dialyzed at about pH 5.7.   To obtain this pH, as much saturated potassium dihydrogen phosphate is added to the solution of catalase as is necessary.   The crystals obtained at the end of about 48 hours, usually of the thin plate type (2), are centrifuged down and are recrystallized in the usual manner.

With alcohol, the same process is repeated except that one substitutes alcohol for acetone.   Here the yield is much lower, as is also the purity of the first crude catalase precipitate.   Moreover, much catalase is found in

TABLE I

Analysis of Crystalline Beef Liver Catalase

Method of preparation	Kat. f.	Iron
		per cent
Acetone.......................	31,900	0.17
Alcohol......................	30,200	0.12

higher and lower fractions precipitated with alcohol.   In this connection it should be pointed out that dioxane precipitates the enzyme sharply, acetone somewhat less sharply, and alcohol much less sharply.   In fact, it is only owing to the extreme case of crystallization of beef liver catalase by dialysis in the presence of impurities that one is able to obtain any crystals at all using the alcohol method.

It is of interest that once recrystallized beef catalase prepared by the use of acetone or alcohol is analytically identical with catalase prepared by the original dioxane method, except for a slight amount of iron-containing impurity, as shown in Table I.   This is not always true when a crystalline protein is prepared by different methods.   The iron-containing impurity no doubt could be removed by one or two further recrystallizations.   Beef catalase prepared through use of acetone or alcohol contains both hematin and the bile pigment-iron complex (3).

The highest yield of crystalline beef liver catalase is obtained through the use of dioxane.   With acetone the yield is satisfactory, but with alcohol it is very low.

SUMMARY

The crystallization and properties of lamb liver catalase are described. Methods are given for the preparation of crystalline beef liver catalase by the substitution of alcohol or acetone for the solvent dioxane, originally employed. Catalase prepared by using alcohol or acetone is practically identical with that obtained by the dioxane method.

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AUTOXIDATION OF STEROLS IN COLLOIDAL AQUEOUS SOLUTION

II. Δ⁶-CHOLESTENEDIOL-3(β),5, A REARRANGEMENT PRODUCT OF 7(β)-HYDROXYCHOLESTEROL

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In a recent paper (1) we described the isolation of a new cholestenediol, then provisionally named Compound A, from the products which are formed on aeration of an aqueous colloidal solution of cholesterol. We furthermore showed that this compound is formed from 7(β)-hydroxycholesterol, which is probably the primary oxidation product, by treatment with Girard's reagent, or merely by boiling with alcohol containing acetic acid. The properties of the diol suggested that it was a Δ⁶-cholestenediol-3,5 which had arisen from the parent substance by an allylic rearrangement. Evidence is now presented to show that the provisionally assigned structure is correct.

First we attempted to reduce the compound catalytically or with nascent hydrogen to cholestanediol-3,5. However, in a neutral medium the diol failed to take up hydrogen with any of the catalysts tried (platinum oxide, palladium, Raney's nickel). Treatment with sodium in boiling *n*-propyl alcohol likewise left the compound unchanged. On catalytic reduction in acetic acid, on the other hand, 2 moles of hydrogen were absorbed rapidly and without recognizable break in the rate after uptake of 1 mole. In contradistinction to the starting compound, the reduced product was for the most part precipitable by digitonin. Decomposition of the digitonide yielded a product melting at 110–120°, which had the composition $C_{27}H_{48}O$. Obviously the tertiary hydroxyl group had been reductively removed, a reaction not uncommon with allylic sterol alcohols. The product could not be purified by crystallization and was therefore subjected to the epimerization procedure of Windaus (2) and subsequent separation with digitonin, a method which had been used by Schoenheimer and Evans (3) for identifying the reduction products from allocholesterol and epiallocholesterol. The digitonin-precipitable portion yielded β-cholestanol (dihydrocholesterol), while from the non-precipitable fraction α-coprostanol (epicoprostanol) was isolated. As β-coprostanol is epi-

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merized to the extent of 90 per cent, whereas β-cholestanol yields under the same conditions only a small amount of its epimer, the original digitonin-precipitable fraction must have consisted of a mixture of β-cholestanol and β-coprostanol. The two 5-epimeric compounds may have arisen, as in the case of allocholesterol (3), by the reduction of a 4-5 double bond, formed from the 5-hydroxyl group by dehydration. The intermediary formation of a Δ⁵-compound after saturation of the original double bond, though less likely, is not necessarily contradicted by our result, since reduction of a 5-6 double bond may also yield as by-products compounds with the coprostane configuration at C_5 (4).

The location of the second, non-esterifiable hydroxyl group and of the double bond in the new diol was proved by oxidation to the Δ⁴,⁶-cholestadienone-3 first prepared by Dane, Wang, and Schulte (5). The chromic acid method proved unsuitable for this purpose, no matter whether the diol was oxidized as such or after protection of its double bond with bromine. In either case practically all the material was recovered in the neutral fraction, but even after fractionation by the chromatographic method no crystallizable products could be isolated. Better success was attained with the method of Oppenauer as modified by Kuwada and Toyama (6) and by Reich and Reichstein (7), entailing the use of aluminum phenolate instead of aluminum tert-butylate. The oxidation product was identified as Δ⁴,⁶-cholestadienone-3 by comparison with a preparation made by the method of Dane et al. (5). There was no indication of the presence in the oxidized material of the keto alcohol which must have been the primary reaction product. The result leaves little doubt that the tertiary hydroxyl group in the diol is located in position 5, and that the original double bond occupies the 6-7 position. The elimination of the tertiary hydroxyl group to form the 4-5 double bond is expected to occur under the tendency to form a triply conjugated system after establishment of the keto group at C_3.

The possibility that a double bond migration may have taken place during the oxidation is extremely remote. Nevertheless it seemed desirable to adduce additional structural proof by reactions in which the 3-hydroxyl group was not involved. Thus the elimination of the elements of water from C_4 and C_5 should lead to Δ⁴,⁶-cholestadienol-3(β), which is known to be stable in the form of its esters, but so far we have been unable to effect this conversion. Boiling with acetic anhydride resulted in partial decomposition, and no crystalline product could be isolated. The absorption spectrum of the reaction product indicated that it contained a triene. Chlorinating agents, on the other hand, left the tertiary hydroxyl group intact. The benzoate of the diol was recovered quantitatively after

treatment with thionyl chloride in pyridine at 0° (Darzen's reaction), while at elevated temperature (70–80°) partial decomposition took place. Phosphorus oxychloride in pyridine, used successfully by Butenandt, Schmidt-Thomé, and Paul (8) for the dehydration of tertiary hydroxyl groups at C_{17}, likewise left the ester unchanged. The inertness of the diol benzoate towards thionyl chloride is surprising in view of the behavior of the diacetate of α-cholestanetriol-3,5,6, which could be smoothly converted into Δ^4-cholestenediol-3,6 diacetate by the Darzen reaction (9). This difference in reactivity suggests that the two compounds may carry the 5-hydroxyl group in opposite steric arrangement.

EXPERIMENTAL

Compound A was prepared by rearrangement of 7(β)-hydroxycholesterol with boiling alcohol containing 10 per cent of acetic acid, as described in our previous paper (1). Various modifications, such as increasing the proportion of acetic acid, or substituting hydrochloric acid for the latter, were tried, but in most cases the yield did not approximate that given by the original procedure. Since purification by recrystallization leads to considerable losses, the reaction product was acetylated, adsorbed in pentane solution on aluminum oxide, and eluted as described for the separation of the chromogen fraction of autoxidized cholesterol (1). Saponification of the fractions with $[\alpha]_D > -100°$ and crystallization from methanol yielded Compound A, melting at 137–138°.

Hydrogenation—186 mg. of the diol were dissolved in 5 cc. of glacial acetic acid and shaken with 20 mg. of platinum oxide (Adams-Shriner) in an atmosphere of hydrogen. The uptake of hydrogen stopped after 5 hours, when 2 moles had been consumed. The filtered solution was diluted with ether, washed with sodium carbonate solution and water, and taken to dryness. The residue was treated with digitonin (1 gm. in 100 cc. of 80 per cent ethanol), yielding 600 mg. of digitonide. On decomposition of the precipitate with pyridine 135 mg. of a crystalline product melting at 110–120° were recovered. The mixture could not be separated by fractional crystallization. Analysis showed that it contained only saturated monohydric sterols.

Analysis—$C_{27}H_{48}O$. Calculated. C 83.44, H 12.45
Found. " 83.85, " 12.55

The material was then epimerized (2) by boiling it for 8 hours with xylene (25 cc.) and sodium (6 gm.). The resulting product was separated in the usual way by means of digitonin. The digitonide (252 mg.) on decomposition yielded 58 mg. of colorless needles which after two crystalli-

zations from methanol melted at 140–141°.[1]   A mixture with β-cholestanol melted at the same temperature.

Analysis—C$_{27}$H$_{48}$O.   Calculated.   C 83.44, H 12.45
                    Found.   " 83.59, " 12.38
                    $[\alpha]_D^{23} = +23°$

The filtrate from the digitonide was brought to dryness and extracted with ether.   The resulting oil (47 mg.) was dissolved in 20 cc. of benzene and adsorbed on a column of aluminum oxide.   Elution was effected with 20 cc. portions of benzene-ether 9:1 and of ether.   The ether solution yielded 14 mg. of a crystalline material which after recrystallization melted at 115–116°.   The melting point was not depressed in mixture with pure α-coprostanol.

Analysis—C$_{27}$H$_{48}$O.   Calculated.   C 83.44, H 12.45
                    Found.   " 83.70, " 12.88

Conversion to Δ^{4,6}-*Cholestadienone-3*—The diol (200 mg.) was boiled for 12 hours with a mixture of dry benzene (50 cc.) and freshly distilled acetone (12 cc.) containing 1.5 gm. of aluminum phenolate (6, 7).   After the addition of ether the solution was washed successively with dilute hydrochloric acid, sodium hydroxide, and water, and evaporated to dryness.   The residue, which no longer gave the Lifschütz reaction, was dissolved in 40 cc. of petroleum ether and adsorbed on a column of aluminum oxide. Elution with 40 cc. portions of solvents yielded the following fractions: Fraction 1, petroleum ether, none; Fraction 2, petroleum ether-benzene 1:1, 5 mg.; Fractions 3 and 4, benzene, 47, 89 mg.; Fraction 5, benzene-ether 1:1, 48 mg.; Fraction 6, ether, 4 mg.   Fraction 3 was refluxed for 3 hours with an excess of hydroxylamine acetate in 90 per cent ethanol. The oxime was precipitated with water and taken into ether.   The ether residue was recrystallized three times from ether-methanol and then melted at 174–175°.

Analysis—C$_{27}$H$_{43}$ON.   Calculated.   C 81.55, H 10.91, N 3.52
                    Found.   " 81.47, " 11.00, " 3.73

Fraction 4 was treated with semicarbazide acetate in a similar manner. The crystalline product formed after 1 hour's boiling was filtered off and recrystallized from ethanol.   It melted at 228–230° with decomposition starting at 220°.

Analysis—C$_{28}$H$_{45}$ON$_3$.   Calculated.   C 76.45, H 10.32, N 9.56
                    Found.   " 76.43, " 10.37, " 9.79
                    $\epsilon_{305m\mu} = 45,000$ (dioxane)

20 mg. of the semicarbazone were boiled for 2 hours in 5 cc. of acetic acid containing 200 mg. of oxalic acid.   The reaction product, isolated by ether extraction, was a yellow oil.   It was purified by adsorption from

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[1] All melting points are corrected.

petroleum ether solution on aluminum oxide. Elution with 15 cc. portions of solvents as described above yielded in the second benzene washing 9 mg. of crystals; one recrystallization from methanol raised the melting point to that of pure $\Delta^{4,6}$-cholestadienone-3 (80–81°). $\epsilon_{285m\mu} = 25,000$ (ethanol).

The identity of the oxime, the semicarbazone, and the free ketodiene was established by determination of the melting points in mixture with pure reference preparations. No depression was observed in any case. $\Delta^{4,6}$-Cholestadienone-3 was prepared from cholesterol dibromide according to Dane et al. (5); m.p. 80–81°; $[\alpha]_D^{25} = +33.4°$ (chloroform).

Analysis—$C_{27}H_{42}O$. Calculated. C 84.65, H 11.07
Found. " 84.75, " 11.21

The ultraviolet absorption characteristics of this preparation and of its semicarbazone checked exactly with those given above for the preparations made from Δ^6-cholestenediol-3,5. In the absorption curves published by Dane et al. the ordinate values are expressed as logarithms of an undefined unit designated E (molar). Calculated as E, these values are 40,000 at 280 mμ for the ketodiene in dioxane and 115,000 at 308 mμ for the semicarbazone in the same solvent. If E (molar) should be represented by $2.303 \times \epsilon$, the corresponding values of ϵ^2 would be 17,400 and 50,000, which are in better agreement with our measurements.

SUMMARY

The new cholestenediol recently isolated from the products formed by autoxidation of cholesterol in colloidal solution has been shown to possess the structure of a Δ^6-cholestenediol-3(β),5. Its precursor is in all probability 7(β)-hydroxycholesterol, from which it is formed *in vitro* by allylic rearrangement under the influence of acid.

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² $\epsilon = 1/l \cdot c \log_{10} I_0/I$; c = concentration in moles per liter.

DISTRIBUTION OF ISOTOPIC NITROGEN IN AZOTOBACTER VINELANDII

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The nitrogen metabolism of rats fed a variety of compounds labeled with N^{15} has been extensively investigated by Schoenheimer and Rittenberg (1) and their associates. Few studies are reported, however, in which plants or bacteria have been used. Vickery, Pucher, Schoenheimer, and Rittenberg (2) have studied the nitrogen metabolism of the tobacco plant with N^{15}. Burris and Miller (3) indicated the applicability of N^{15} as a tracer in biological nitrogen fixation. By furnishing a non-equilibrium mixture of N_2 molecules of mass 28, 29, and 30 to *Azotobacter vinelandii*, they established that N^{14} and N^{15} were fixed indiscriminately and that no exchange reaction occurred between molecular nitrogen and fixed nitrogen in the culture.

Application of the stable isotope of nitrogen, N^{15}, should clarify many problems in biological nitrogen fixation. The present communication, dealing with the distribution of the isotope among nitrogenous compounds formed by *Azotobacter vinelandii* from N^{15} supplied as molecular nitrogen, is an example of such an application.

EXPERIMENTAL

Azotobacter vinelandii cultures were grown in Burk's (4) nitrogen-free medium with 2.5 per cent sucrose. These liquid cultures were incubated at 30° and aerated vigorously for a period of 18 hours, at which time they had fixed approximately 10 mg. of nitrogen per 100 ml. of medium. The 18 hour cultures were transferred to a closed circuit aerating system (Fig. 1) which was arranged to circulate continuously a gas mixture through the cultures. The culture bottles were attached by rubber tubing at points *1* and *2*. Cotton plugs *3* and *4* served to filter the gas during both the initial and final aerations. The entire system was evacuated through stop-cock *5*, and a gas mixture consisting of 40 per cent tank oxygen and 60 per cent nitrogen containing 35 atom per cent N^{15} excess was introduced through stop-cock *6* until the mercury manometer *7* registered zero vacuum. A

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dry ice trap *8* prevented mercury vapor from entering the system from the manometer. A rubber tube pump *9* of the Urey type, which circulates liquid or gas by compression of a section of rubber tubing, circulated the gas mixture at a rate of approximately 600 ml. per minute. The gas was broken into fine bubbles by an aeration stone *10*. Passage of the gas through a soda lime tube *11* removed the carbon dioxide produced by the bacteria; at intervals tank oxygen was added through stop-cock *5* to replace the oxygen used by the organisms. The culture bottle was kept in a water bath at 30°.
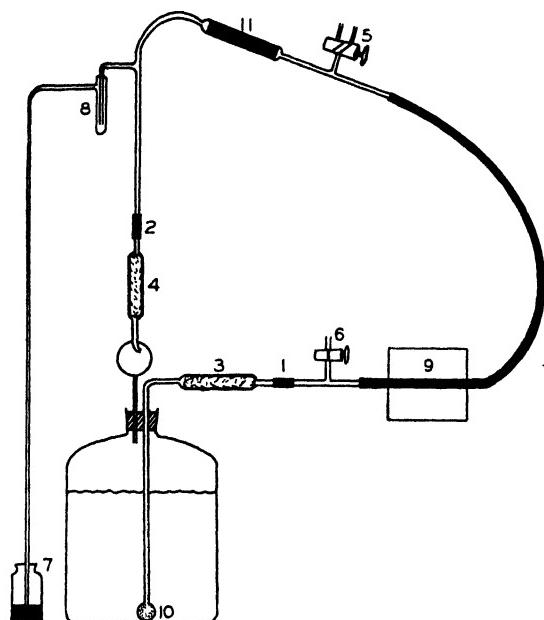
FIG. 1. Closed circuit aerating system for continuous circulation of a gas mixture through bacterial cultures.

After the oxygen and isotopic nitrogen mixture had been circulated for 90 minutes, a pinch-clamp at *1* was closed and the gas was recovered by pumping it into an evacuated bulb attached at *6*. The culture was placed in an ice bath to decrease metabolism, and as quickly as possible the bacterial cells were centrifuged from the medium with a Sharples supercentrifuge driven at 40,000 R.P.M. The centrifuge bowl had been previously chilled. The cells scraped from the bowl were transferred to 8 N sulfuric acid and quickly heated to boiling temperature. Eight 800 ml. cultures were treated in this manner. The average time from the cessation of aeration to the immersion of the cells in acid was 30 minutes.

Cells from the eight cultures were combined and hydrolyzed for 24 hours in 8 N sulfuric acid. The humin was filtered off and the sulfate removed by the addition of barium hydroxide. Analysis for N^{15} with a mass spectrometer of the Bleakney type indicated that the total hydrolysate at this point carried 0.275 atom per cent N^{15} excess. Amino acids of the hydrolysate were precipitated with Neuberg's (5) reagent and the washed precipitate decomposed with hydrogen sulfide. The solution from the decomposition of the Neuberg precipitate contained 410 mg. of nitrogen. From this solution amino acids and amino acid fractions were separated.

Arginine—The amino acid solution yielded 950 mg. of arginine flavianate on the first crystallization, which is equivalent to 109.0 mg. of arginine nitrogen. The arginine flavianate was recrystallized, and 715 mg. of recrystallized material were decomposed with 10 ml. of cold concentrated hydrochloric acid. The flavianic acid was filtered off and the arginine solution was diluted, treated with activated carbon, filtered, and analyzed for its nitrogen content. The arginine solution, which contained 57 mg. of nitrogen, was treated with *p*-toluenesulfonyl chloride. The *p*-toluenesulfonylarginine derivative was crystallized four times. The melting point observed was 256–257°, correct melting point 256–257°. Nitrogen, found 16.67 per cent; theory 17.07 per cent. Isotope analysis, 0.185 atom per cent N^{15} excess.

To 20 ml. of the arginine solution (12.75 mg. of N) derived from the decomposition of arginine flavianate 5 gm. of barium hydroxide were added. The solution was refluxed for 24 hours while a slow stream of nitrogen swept the liberated ammonia into dilute sulfuric acid. This ammonia, arising from the guanidino group of arginine, contained 0.212 atom per cent N^{15} excess, a value but slightly greater than that for the total arginine nitrogen. Calculation indicates that the N^{15} concentration in the ornithine portion of arginine would be 0.158 atom per cent N^{15} excess.

"Amide" Nitrogen—The filtrate from the precipitation of arginine flavianate was brought to pH 6.5, filtered, and the precipitate washed. After solid barium hydroxide had been added to make the filtrate and washings alkaline to phenolphthalein, the solution was distilled *in vacuo* at 35° for 2 hours. The ammonia nitrogen liberated (20.4 mg.) was trapped in dilute sulfuric acid; this "amide" nitrogen contained 0.325 atom per cent N^{15} excess.

Dicarboxylic Amino Acids—After the removal of "amide" nitrogen, the solution was filtered, and 4 volumes of ethyl alcohol were added to precipitate the barium salts of the dicarboxylic acids. The precipitate was washed, dissolved in water, filtered, and reprecipitated with alcohol. The reprecipitated material was dissolved and the barium was removed with sulfuric acid. The solution of dicarboxylic acids (36.4 mg. of N), reduced to 2 ml.

volume by vacuum distillation, was saturated with hydrogen chloride gas to precipitate glutamic acid hydrochloride. After four crystallizations the glutamic acid hydrochloride melted at 202–203° with decomposition. Nitrogen, found 7.55 per cent; theory 7.63 per cent. Isotope analysis, 0.500 atom per cent N^{15} excess.

The glutamic acid hydrochloride filtrate was freed of hydrochloric acid by vacuum distillation. Excess cupric carbonate was added to the boiling solution; the hot solution was filtered. Crystals of copper aspartate failed to appear even after reduction of the volume of the filtrate to 2 ml., seeding, and refrigeration for 10 days. Analysis of an aliquot showed 0.376 atom per cent N^{15} excess.

Histidine—The filtrate and washings from the precipitation of the dicarboxylic amino acids were treated with sulfuric acid to remove barium, and concentrated by vacuum distillation. Basic amino acids were precipitated from acid solution with phosphotungstic acid. The precipitate was decomposed with a butyl alcohol-ethyl ether mixture, concentrated by vacuum distillation to remove butyl alcohol, and filtered. Histidine was precipitated as the mercury salt at pH 7.0 to 7.5, refrigerated, filtered, washed, and decomposed with hydrogen sulfide. Histidine monohydrochloride crystallized from a small volume upon the addition of ethyl alcohol and acetone. Isotope analysis, 0.207 atom per cent N^{15} excess.

Lysine Fraction—The filtrate from the precipitation of the mercury salt of histidine contained 12.7 mg. of N containing 0.356 atom per cent N^{15} excess.

Monoamino Acids—The filtrate from the phosphotungstic acid precipitation was freed of phosphotungstic acid, and copper salts were formed of the amino acids in the filtrate. The salts were dried by vacuum distillation, desiccated, powdered, triturated several times with small portions of cold water, and filtered after each trituration. The water-soluble copper salts were again dried by vacuum distillation and desiccation, triturated several times with absolute methyl alcohol, and filtered. The three copper salt fractions were suspended in weak hydrochloric acid and decomposed with hydrogen sulfide.

From the data of Town (6) it appears that these copper salt fractions may contain the following amino acids: *water-insoluble*, leucine, phenylalanine, tyrosine, and cystine; *water-soluble* and *methyl alcohol-insoluble*, glycine, serine, and alanine; *water-soluble* and *methyl alcohol-soluble*, proline, hydroxyproline, isoleucine, and valine. The isotopic analysis indicated that all three fractions contained about 0.32 atom per cent N^{15} excess.

A summary of the distribution of N^{15} and total nitrogen in fractions from the Neuberg precipitate is given in Table I.

Neuberg Filtrate—The filtrate from the precipitation of amino acids with

Neuberg's reagent contained 14.7 mg. of nitrogen; isotope analysis, 0.441 atom per cent N^{15} excess. The precipitate of purines and pyrimidines formed with silver acetate contained 0.262 atom per cent N^{15} excess. A quantitative ninhydrin reaction indicated that 65 per cent of the nitrogen in the filtrate from the silver salt precipitation was α-amino nitrogen. No urea was precipitated with xanthydrol.

Supernatant from Azotobacter Culture—A sample of the supernatant material which was separated from the *Azotobacter* cells in the centrifuge contained 0.073 atom per cent N^{15} excess.

TABLE I

Distribution of N^{15} and Total Nitrogen in Neuberg Precipitate

The Neuberg precipitate contained 410 mg. of N before fractionation.

	N content	Per cent total N	N^{15} excess	Per cent total N^{15} excess recovered
	mg.		*atom per cent*	
Arginine	109.0	26.60	0.185	20.20
"Amide" nitrogen	20.4	4.98	0.325	6.63
Glutamic acid.	16.7	4.07	0.500	8.35
Aspartic " fraction . .	19.5	4.77	0.376	7.33
Histidine..	54.9	13.40	0.207	11.38
Lysine fraction	12.7	3.10	0.356	4.52
H_2O-insoluble Cu salts	32.6	7.95	0.313	10.20
H_2O-soluble MeOH-insoluble Cu salts.	19.4	4.73	0.320	6.20
" MeOH-soluble Cu salts . .	77.2	18.83	0.327	25.15
Total	362.4	88.43		

DISCUSSION

Knowledge of the nitrogen metabolism of nitrogen-fixing bacteria and plants is very sketchy because investigators have lacked a suitable means of tracing the element after its fixation. Although the results reported in this paper do not establish a mechanism for the process, they do afford criteria for judging the validity of proposed mechanisms. By halting quickly the metabolism of *Azotobacter* cells which have fixed molecular N^{15} for a short time after an initial period of fixation in air, we have obtained organisms in which the N^{15} has not come to equilibrium with the N^{14} fixed earlier. The levels of N^{15} in the various fractions indicate which of these first acquire the nitrogen fixed by the organism, for, unless we accept the unlikely assumption that amino acids are formed at a different rate at different culture ages, the level of N^{15} measures the rate of formation of amino acids from *recently* fixed nitrogen.

The traditional view of biological nitrogen fixation assumes that ammonia is an intermediate. Of late Winogradsky (7) has strongly championed this scheme, but as Wilson (8) has emphasized, the experimental evidence in support is hardly critical. During the past decade Virtanen (9) has proposed that biological nitrogen fixation proceeds via hydroxylamine, as suggested earlier by Blom (10). Virtanen has provided the only real experimental evidence and has greatly extended the details of the mechanism. He believes that the hydroxylamine combines with oxalacetic acid to yield oximinosuccinic acid which is then reduced to aspartic acid. His finding that the material excreted from the nodules of inoculated pea plants is primarily aspartic acid constitutes at best only indirect support for the scheme, since formation of this compound does not necessarily require hydroxylamine as a precursor. His report that he has isolated small quantities of oximinosuccinic acid from the excretion products provides much more impressive evidence, since the formation of this compound involves hydroxylamine directly (8).

What are the relevant data in Table I with respect to these views of the mechanism? First it should be noted that glutamic acid apparently occupies a key position in the nitrogen metabolism of *Azotobacter*, since it carries the highest level of N^{15} of any compound isolated. It is likely that α-ketoglutaric acid accepts fixed nitrogen to form glutamic acid and the latter in turn transfers this nitrogen to carbon chains in the formation of other amino acids. Second, the rôle of aspartic acid cannot be defined with certainty, since no pure derivative could be isolated and analyzed for its isotopic concentration. Although it is impossible to state whether the value 0.376 atom per cent N^{15} excess found in the aspartic acid fraction represents the N^{15} level in the aspartic acid present, it scarcely seems probable that the value would be higher, since the chief contaminant in the fraction would be glutamic acid with a high N^{15} value. In a separate experiment fractionation of a hydrolysate of *Azotobacter* cells containing 5840 mg. of normal nitrogen yielded 1.11 per cent of the total nitrogen as aspartic acid nitrogen (copper salt isolated) and 5.54 per cent as glutamic acid nitrogen (hydrochloride isolated). Thus glutamic acid is present in 5-fold greater concentration than aspartic acid in *Azotobacter*. Finally, a surprisingly high level of N^{15} was found in the Neuberg filtrate, 0.441 atom per cent N^{15} excess. In view of this high value it is not unlikely that some intermediate with a very high level of N^{15} occurred in small quantities in this filtrate, since one would expect the level of N^{15} in any particular compound in this fraction to be diluted by the variety of compounds present.

Although these data certainly do not allow a decision to be made regarding the plausibility of either the hydroxylamine or ammonia hypothesis, certain conclusions are suggested. First, the high level of N^{15} in glutamic

acid might be interpreted as support for the ammonia hypothesis. Virtanen (11) states that, whereas ammonia reacts with equal rapidity with α-ketoglutaric acid and oxalacetic acid, hydroxylamine reacts much more rapidly with the latter. He argues from this that glutamic acid should be found in the excreted products if ammonia were an intermediate. Reversing the argument, one might conclude that finding a high level of N^{15} in both glutamic and aspartic acids results from α-ketoglutaric and oxalacetic acids acting as acceptors for ammonia formed as an intermediate.

The fact that in rats (12, 13) and tobacco plants (2) given N^{15}-enriched ammonium ion glutamic acid has a consistently higher level of the heavy isotope than has aspartic acid likewise suggests that α-ketoglutaric acid is the first nitrogen acceptor and that the relatively high level of N^{15} in aspartic acid can be attributed to the much greater speed of transamination to oxalacetic acid than to other compounds (14).

What might be interpreted as conflicting with the ammonia hypothesis, however, is the observation that the level of N^{15} in "amide" nitrogen is but slightly higher than the average for the entire hydrolysate. Tobacco plants supplied with ammonium ion enriched with N^{15} possess a higher N^{15} level in "amide" nitrogen than in any other organic fraction isolated (2). The same is true of the total carcass of immature rats on a protein-free, ammonia-rich diet (12), but not for the "amide nitrogen of protein" from mature rats on a normal diet (13). These results, however, do not necessarily oppose the ammonia hypothesis, since no evidence exists that excess ammonia is stored as amide in *Azotobacter*, as is believed to be the case for many plants. Moreover, there is no reason to believe that, in bacteria fixing nitrogen under the conditions used here, ammonia would be in excess sufficient to require storage in an amide reservoir. It is planned to study the ammonia metabolism of *Azotobacter* by use of N^{15} ammonia.

Another possible mechanism was suggested by the work of Orcutt (15), who demonstrated that basic non-amino nitrogen in inoculated soy bean plants entering the period of active nitrogen fixation was higher than in control plants furnished combined nitrogen. He suggested that a compound containing basic non-amino nitrogen, possibly arginine, was involved in the fixation process. Umbreit and Burris (16) fractionated the soluble nitrogen compounds in soy bean nodules and found a high level of a compound which readily lost ammonia in the presence of alkali and was tentatively identified as arginine. Although arginine nitrogen constitutes more than one-quarter of the total amino acid nitrogen in the *Azotobacter* cells studied, the low level of N^{15} found in this fraction argues against an "arginine hypothesis" of nitrogen fixation. In rats fed N^{15}-enriched ammonium citrate (12) only traces of the heavy isotope were found in the ornithine portion of arginine, whereas a level about the same as that in aspartic acid

appeared in the guanidino group. *Azotobacter* synthesizes the complete arginine molecule and the N^{15} level of the guanidino nitrogen is but slightly higher than that in the ornithine part of the molecule. This observation conflicts with an arginine hypothesis which would ascribe particular activity to the guanidino grouping.

In addition to the observations whose primary significance relates to their bearing on the mechanism of the fixation reaction, a few others of more general import may be mentioned. The supernatant culture medium separated from the cells by centrifugation contained 0.073 atom per cent N^{15} excess, a value about one-fourth the N^{15} concentration within the bacterial cells. Evidently during the 90 minute aeration with molecular N^{15} the nitrogen compounds inside the cells did not come to equilibrium with those in the external medium.

Although our nitrogen fractionation was not primarily designed to provide a quantitative nitrogen distribution, the data are nevertheless of interest from this point of view. The fractions listed account for 88.4 per cent of the total nitrogen. Greene (17) reported nitrogen distribution (Van Slyke) on four species of *Azotobacter*. *Azotobacter vinelandii* yielded the following values for per cent total nitrogen: humin, 15.8, arginine 16.7, histidine 0.16, cystine 0.29, lysine 5.1, filtrate amino nitrogen 39.3, and filtrate non-amino nitrogen 2.25. Comparison with our values, Table I, second column, indicates rather wide discrepancies, especially with regard to arginine, "amide" and histidine nitrogen. Since we have isolated 26.6 per cent of the total nitrogen as arginine, it seems possible that Greene's treatment to remove "amide" nitrogen partially decomposed the arginine present, thus giving a low value for the basic nitrogen. The Van Slyke distribution evidently did not properly distinguish histidine and lysine.

It is of particular interest that, of the amino acids and amino acid fractions isolated from *Azotobacter* cells fixing molecular N^{15}, the highest levels of the isotope should occur in glutamic and aspartic acids, with low levels in arginine and histidine, for when ammonium citrate or amino acids labeled with N^{15} are fed to rats (1, 12, 13) or when ammonium chloride is furnished the tobacco plant (2), the highest levels of N^{15} in the amino acids (exclusive of the compound administered) are invariably found in glutamic and aspartic acids. Likewise arginine and histidine have low levels of N^{15}. There thus appear to be striking similarities in the nitrogen metabolism of widely separated biological species metabolizing nitrogen compounds ranging from molecular nitrogen to amino acids. That glutamic and aspartic acids should play important metabolic rôles is quite in harmony with the demonstration by Cohen (14) that these compounds are by far the most active amino acids in the process of transamination.

SUMMARY

Azotobacter vinelandii cells supplied with molecular N^{15} for 90 minutes after an initial period of 18 hours growth in normal nitrogen were fractionated to determine the distribution of total nitrogen and isotopic nitrogen. Arginine and histidine were present in large quantities but carried low levels of N^{15}. "Amide" nitrogen and three copper salt fractions of the mono-amino acids gave intermediate values for the N^{15} isotope, whereas the lysine fraction was somewhat higher in N^{15}. Among the amino acids, glutamic acid and the aspartic acid fraction showed the highest levels of N^{15}. The Neuberg filtrate was high and the extracellular nitrogen of the culture was low in isotope concentration.

The data are discussed in relation to proposed mechanisms of biological nitrogen fixation.

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OCCURRENCE IN NATURAL PRODUCTS OF A PHYSIOLOGI-
CALLY ACTIVE METABOLITE OF PYRIDOXINE

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Möller (1, 2) first showed that a variety of lactic acid bacteria required pyridoxine for growth on a chemically defined medium free of this substance. He further showed that the synthetic and the isolated vitamins were qualitatively and quantitatively identical in their growth effect on these organisms.

Since these initial findings, several other microorganisms have been shown either to require pyridoxine for growth, or to be greatly stimulated by its presence under defined conditions. Certain hemolytic streptococci (3, 4) belong in this first class, while various yeasts (2, 5) belong in the second class.

While pyridoxine, isolated or synthetic, thus demonstrably promotes growth of such organisms in media deficient in this substance alone, it has not been generally demonstrated that growth increments obtained on addition of tissue extracts to such media are due only to the pyridoxine content of the extracts. Such a demonstration is required before an organism can be employed for the biological assay of a vitamin.

Williams, Eakin, and McMahan (6) have recently presented an assay method for pyridoxine, based on stimulation of yeast growth, which appears specific. Assay values obtained by its use are, however, considerably lower than assays obtained on corresponding samples by the rat growth method of Conger and Elvehjem (7). In an attempt to devise an independent microbiological assay for pyridoxine, the response of one of the lactic acid bacteria, *Streptococcus lactis* R, to the vitamin was investigated. This organism grew luxuriantly through repeated subculture when pyridoxine was added to the base medium, but growth failed completely in the absence of pyridoxine or some organic extract. Assay of such extracts for their apparent pyridoxine content by use of this organism gave values which ranged from several hundred to several thousand times accepted values for such materials, whether the latter values be obtained by yeast growth, rat growth, or colorimetric (8) methods.

Two explanations present themselves: (*a*) the organism, in addition to responding to pyridoxine, was responding to a substance (or substances) present in the extracts which differed from pyridoxine and which either greatly surpassed pyridoxine in physiological activity, or was present in

considerably greater quantity; or (b) a substance (or substances) is present in tissue extracts which, though inactive alone, greatly increased sensitivity of *Streptococcus lactis* to pyridoxine.

Preliminary fractionation experiments showed this substance responsible for the extra response to be closely similar to pyridoxine. Thus on fractional electrical transport in the apparatus described by Williams (9) the active substance migrated to the same cell as did pyridoxine. Both substances were adsorbed by fullers' earth, by charcoal, and by the synthetic zeolite Decalso, and elution of both substances was effected by the same general procedures. Both substances showed similar stability to acid and alkali treatment.

The experiments described below indicate that this substance is a naturally occurring metabolic product which may arise from pyridoxine itself, and which far surpasses pyridoxine in physiological activity for *Streptococcus lactis*.

<div align="center">EXPERIMENTAL</div>

Testing Procedures—Pyridoxine assays were obtained by the yeast growth method (6). The effect of pyridoxine or of the tissue extract factor which replaces pyridoxine on *Streptococcus lactis* R (American Type Culture Collection No. 8043) was measured on the medium described in Table I.

Acid-hydrolyzed, charcoal-treated casein for Solution 1 is prepared by refluxing 50 gm. of Labco vitamin-free casein with 500 cc. of constant boiling HCl for 8 to 10 hours. Excess HCl is removed by repeated concentrations *in vacuo*. The resulting syrup is dissolved in water, adjusted to pH 3.0, and shaken for 10 minutes with 1 part of activated charcoal (Darco G-60) to 10 parts of solids, and then filtered.

Assays are carried out in test-tubes. For the standard curve, tubes containing from 0 to 3.0 γ of pyridoxine are prepared. Various amounts of tissue extract are selected by experiment which are approximately equivalent in growth effect to these amounts of pyridoxine. All tubes are then diluted to 5 cc. with water, and 5 cc. of the base medium added. The tubes are plugged with cotton, and then sterilized at 15 pounds of steam pressure for 10 minutes. After cooling to room temperature, they are ready for inoculation. Cells for inoculum are prepared by transfer from a stab culture of the test organism to a tube containing 10 cc. of base medium supplemented with 3 γ of pyridoxine. This culture is incubated at 30° for from 16 to 30 hours before use. The cells are centrifuged out aseptically, then resuspended in 10 cc. of 0.9 per cent NaCl solution. An aliquot of this suspension is diluted to barely visible turbidity. 1 drop of the diluted suspension is used to inoculate each assay tube. The tubes

are then incubated at 30° for a growth period of 16 hours. Turbidities of the resulting cultures are determined quantitatively with a suitable thermoelectric or photoelectric turbidimeter.

For convenience, in this article the substance present in tissue extracts (in addition to pyridoxine) which replaces pyridoxine for *Streptococcus lactis* will be expressed in terms of the number of micrograms of pyridoxine

TABLE I

Pyridoxine-Free Base Medium for Streptococcus lactis R

				Amount for 50 assay tubes
I	Solution 1			250 cc.
	Hydrolyzed casein (vitamin-free, charcoal-			
	treated)................................	5	gm.	
	Sodium acetate..............................	6	"	
	Tryptophane................................	0.05	"	
	Cystine (hydrochloride).....................	0.1	"	
	Water to make.	500	cc.	
II	Solution 2 ..			5 "
	Adenine sulfate ..	100	mg.	
	Guanine hydrochloride . .	100	"	
	Uracil.	100	"	
	Water to make	100	cc.	
III	Glucose.			5.0 gm.
IV	Solution 3 .			1 0 cc.
	Thiamine chloride	1	mg.	
	Calcium pantothenate	1	"	
	Riboflavin	1	"	
	Nicotinic acid.	1	"	
	Folic acid concentrate*	0.001	"	
	Biotin. .. .	0.004	"	
	Water to make	20.0	cc.	
V	Inorganic salts†			
	Solution A..			2.5 "
	" B.......................			2.5 "

The medium is adjusted to a final pH of 6.8.
* See Mitchell, Snell, and Williams (10).
† Snell and Strong (11).

to which it is equivalent in growth effect, and will be called pseudopyridoxine. This value is usually sufficiently high so that the actual pyridoxine content of the tissue, which is superimposed in the final growth effect, can be neglected in comparison. *The figures given for the pseudopyridoxine content of a tissue are thus not to be confused with the actual amount of active substance present, which is unknown.*

Sample data from an experiment in which Difco yeast extract was assayed are given in Table II. 1 gm. is equivalent in growth effect to 16,800 γ of pyridoxine. In contrast, this sample of yeast extract contained 15 γ of pyridoxine per gm. by yeast assay, while a value of 40 γ per gm. for another sample of this substance was obtained by Bird *et al.* (12) by a modified Scudi colorimetric procedure. Thus yeast extract is from 400 to 1100 times as potent in promoting growth of *Streptococcus lactis* as can be accounted for on the basis of its pyridoxine content alone.

Pyridoxine and Pseudopyridoxine Content of Tissues from Normal and Pyridoxine-Deficient Rats—Since, as mentioned earlier, no clear cut separation of pseudopyridoxine from pyridoxine could be effected by fractionation of tissue extracts, since electrolysis experiments revealed it to be a base

TABLE II

Assay for Pseudopyridoxine in Yeast Extract

Pyridoxine added per 10 cc. medium	Galvanometer* reading	Yeast extract added per 10 cc. medium	Galvanometer reading	Pseudopyridoxine	Pseudopyridoxine per gm. yeast extract
γ		γ		γ *equivalents*†	γ *equivalents*
0	3.0	0	3.0	0.0	
0.2	9.0	10	8.0	0.15	15,000
0.4	17.0	30	22.0	0.54	18,000
0.7	29.0	50	39.0	0.97	19,500
1.0	40.0	100	54.0	1.48	14,800
2.0	61.0				
3.0	65.0				
Average............					16,800

* Zero reading is 100 per cent transmission; a reading of 100 is complete opacity.

† *Cf.* italicized material in the fourth paragraph under "Experimental" for the significance of these entries in this and succeeding tables.

of the same general strength as pyridoxine, and since both substances promoted growth of the test organism under the same conditions, it was thought possible that the active substance might be metabolically related to pyridoxine.

To test this possibility the following experiment was conducted. Three groups of 21 day-old rats were selected. Each group contained six animals. Group 1 was placed on the pyridoxine-deficient Ration II of Conger and Elvehjem (7). Group 2 was fed the same ration to which were added 3 γ of pyridoxine hydrochloride per gm. Group 3 was maintained on Purina dog chow. All rations were fed *ad libitum*. The average initial weight of all animals was 50 gm. After 35 days on the experimental rations, animals of Group 1 (low pyridoxine) averaged 93 gm. in weight, animals of Group 2 (high pyridoxine) averaged 164 gm., while those in

Group 3 (Purina) averaged 166 gm. At this time two animals of each group were sacrificed. The brain, heart, kidney, liver, and a portion of the leg muscle of each rat were removed. Samples from the two animals of the same group were combined in each case, ground finely, and suspended in dilute acetate buffer (pH 4.5) at a concentration equivalent to 60 mg. of fresh tissue per cc. 1.2 mg. of papain (caroid) and 1.2 mg. of clarase were added per cc. of the tissue suspension, and the mixture incubated under benzene at 37° for 36 hours. This procedure effectively

TABLE III

Comparative Vitamin Content of Rat Tissues from Animals on Low and High Pyridoxine Rations

Substance assayed	Group No.	Pyridoxine	Pseudo-pyridoxine	Ratio, pseudo-pyridoxine to pyridoxine	Pantothenic acid	Biotin
		γ per gm.	γ equivalents per gm.		γ per gm.	mγ per gm.
Ration	1	<0.1	160	>1600		
	2	3.0	160			
	3	0.65	600	920		
Brain	1	0.33	990	3000	9.7	73
	2	3.1	8800	2800	9.7	69
	3	0.54	1640	3000	10.8	100
Heart	1	0.25	470	1900	25.0	110
	2	0.56	2040	3600	24.0	100
	3	0.51	1830	3600	25.0	130
Kidney	1	0.33	520	1600	24.0	580
	2	0.88	3280	3700	31.0	580
	3	0.72	1520	2100	31.0	680
Liver	1	0.27	540	2000	31.0	170
	2	0.84	5600	6400	76.0	580
	3	0.62	1520	2500	69.0	420
Leg muscle	1	0.31	570	1800	6.8	37.0
	2	0.51	1970	3900	6.8	35.0
	3	0.40	1160	2900	6.3	33.0

liberates the "bound" vitamins from their combined forms (13). Samples were then autoclaved at 15 pounds of steam pressure for 15 minutes, cooled, diluted to contain the equivalent of 25 mg. of fresh tissue per cc., filtered, and an aliquot of the filtrate used for assays of pyridoxine and pseudo-pyridoxine. Since it was thought possible that observed differences in the vitamin content of the rat carcasses of Group 1 might reflect only the lowered food intake characteristic of animals of this group, the pantothenic acid and the biotin in the tissues were checked as a control. These were determined by microbiological methods (14, 15).

The results are given in Table III. Without exception, pyridoxine was

lowered in tissues from animals maintained on the pyridoxine-low ration, while tissues from animals on the high pyridoxine ration contained more of the vitamin than those from animals on the dog chow ration. The distribution of pseudopyridoxine paralleled that of pyridoxine; indeed, considerably greater differences in the same direction occur. It should be especially noted that the pseudopyridoxine content of the tissues parallels the pyridoxine content of the ration, and not the pseudopyridoxine content of the ration. The possibility that this effect is due to the presence of a substance not active in the absence of pyridoxine, but greatly increasing the sensitivity of the test organism to pyridoxine already present, may be disposed of, since doubling the pyridoxine content of any of these extracts by addition of synthetic pyridoxine does not change the pseudopyridoxine assay value. Similarly, addition of 3 γ per gm. of synthetic pyridoxine did not raise the pseudopyridoxine assay of the low pyridoxine ration (Table

TABLE IV

Urinary Excretion of Pyridoxine and of Pseudopyridoxine by Rats on Different Diets

Group No.	No. of animals	Total weight of animals	Food consumed, 24 hrs.	Total pyridoxine ingested	Total pyridoxine excreted	Total pseudo-pyridoxine ingested	Total pseudo-pyridoxine excreted
		gm.	*gm.*	γ	γ	γ *equivalents*	γ *equivalents*
1	3	180	3	<0.3	0.07	480	96
2	3	457	23	69.0	4.2	3,680	2480
3	3	529	35	22.8	1.2	21,000	890
1	2	197	5	<0.5	0.21	800	280
2	2	340	18	54.0	2.5	2,880	1600

III). On addition of considerably larger amounts of pyridoxine to such extracts it is quantitatively recovered, but no sensitization to its presence (with consequent recoveries greater than 100 per cent) is observed (*cf.* Table VI). The lowered dietary intake of Group 1 similarly cannot account for the lowered pyridoxine or pseudopyridoxine content of tissues from Group 1, since in general no such effect was observed in the tissue contents of pantothenic acid or of biotin. The lowered content actually observed with liver is in line with present conceptions of this organ as a site of vitamin storage.

Excretion Experiments—After 27 days on the experimental rations, rats from each group were placed in metabolism cages. Records of food intake were kept over the 24 hour period, and the total urinary excretion of pyridoxine and of pseudopyridoxine measured. The results are given in Table IV. In no case did the amounts excreted exceed those ingested. Again, however, rats maintained on the low pyridoxine ration excreted

very little pyridoxine or pseudopyridoxine, while those on the high pyridoxine ration excreted the largest amounts of both substances.   Acid or alkali hydrolysis of these urines did not raise the assay value for either substance. Although animals on the Purina ration ingested 6 times as much pseudopyridoxine as did those on the high pyridoxine ration, their excretion of this substance was only about one-third as high.   This again indicates that animal passage converts part of the ingested pyridoxine to the more highly active (for *Streptococcus lactis*) pseudopyridoxine.

TABLE V

Excretion of Pyridoxine and of Pseudopyridoxine by Male Adult Following Administration of Test Dose of Pyridoxine Hydrochloride

Time after ingestion	Volume of sample	Sample* treatment	Total pyridoxine	Total pyridoxine + pseudo-pyridoxine	Total pseudo-pyridoxine	Ratio, pseudo-pyridoxine to pyridoxine
hrs.	*cc.*		γ	γ *equivalents*	γ *equivalents*	
0 (Control)	34	U.	0.82	320	319	390
		H.	2.08	1,770	1,770	850
1.67	115	U.	34,000	258,000	224,000	6.5
		H.	43,200	273,000	230,800	5.4
2.67	138	U.	15,500	173,000	157,500	10.2
		H.	17,700	203,000	185,300	10.5
6.25	182	U.	7,300	336,000	328,700	45.1
		H.	12,000	380,000	368,000	30.7
8.0	148	U.	370	51,800	51,400	139
		H.	1,200	69,600	68,400	57
12.0	168	U.	270	50,400	50,100	186
		H.	1,200	69,000	67,800	56.6

* U. = untreated, H. = acid-hydrolyzed.   Hydrolysis was carried out with an equal volume of 6 N HCl at 115° for 20 minutes (*cf.* (16)).

An excretion experiment was carried out on a human male subject along lines suggested by recent experiments of Scudi *et al.* (16).   No food was taken on the day of the experiment until after its conclusion.   The morning sample of urine was discarded.   1 hour later, a control sample of urine was taken.   Immediately thereafter 0.5 gm. of pyridoxine hydrochloride was ingested with 300 cc. of water.   Urine samples were taken at intervals for assay.   The results are given in Table V.   Assay values for both pyridoxine and pseudopyridoxine were increased by acid hydrolysis.

Excretion of pyridoxine was most rapid during the 1st hour, and rapidly fell to low levels. The total excretion during the test period was about 15 per cent of the test dose. This is in good agreement with earlier data (16, 17). Excretion of pseudopyridoxine lagged considerably behind that of pyridoxine, so that about 40 per cent of the total amount excreted appeared from the 3rd to the 6th hour. During the corresponding period only 16 per cent of the total excreted pyridoxine appears. This lag is better illustrated by the ratios given in the last column of Table V. The experiment again indicates that pyridoxine is partially converted by the animal body into a product having much greater activity in promoting growth of *Streptococcus lactis* than does pyridoxine itself, and that the relative amount of the conversion product present is greater the longer

TABLE VI

Recoveries of Added Pyridoxine in Presence of Tissue Extracts by Streptococcus lactis

Sample	Pyridoxine present	Pseudo-pyridoxine present	Extra pyridoxine added	Pyridoxine + pseudo-pyridoxine found	Pyridoxine + pseudo-pyridoxine calculated	Per cent of calculated found
	γ per cc. or gm.	γ equivalents per cc. or gm.	γ per cc. or gm.	γ equivalents per cc. or gm.	γ equivalents per cc. or gm.	
Urine I	0.036	6 6	0.04	6.6	6.64	99.4
			250	241	257	93.8
" II	280	2960	250	3120	3210	97.2
Liver I*.	0.23	740	0.3	700	740	93.2
" II*	0.72	2000	0.3	2000	2000	100

* Liver I was from an animal of Group 1 (low pyridoxine diet); Liver II was from an animal of Group 2 (high pyridoxine diet).

pyridoxine remains in the body. The fact that such a lag occurs is additional evidence that the pseudopyridoxine effect is not due to substances which sensitize the organism to unchanged pyridoxine.

Further evidence on this point is given in Table VI. Urine I was a control sample from a second excretion experiment taken before ingestion of 0.5 gm. of pyridoxine hydrochloride. Urine II was a sample taken 3 hours later. Pyridoxine added to these samples or to liver extract samples in approximately the amounts already present did not increase the pseudopyridoxine content above the level calculated to result from such additions. It may be concluded, therefore, that the substance designated throughout this paper as pseudopyridoxine is not a substance which increases the response of the test organism to unchanged pyridoxine already present, but is rather a true metabolic product formed from pyridoxine in the animal body, and occurring together with pyridoxine in natural extracts of plant origin. To account for the data satisfactorily, this product must be con-

siderably more active in promoting growth of *Streptococcus lactis* than is the pyridoxine from which it is derived.

Utilization of Pyridoxine by Streptococcus lactis—The concentration of pyridoxine required to promote maximum growth of *Streptococcus lactis* (*cf.* Table III) is considerably higher than the amount of other similar accessory growth substances required by this or by related organisms. It seems likely that in the range in which the concentration of an accessory substance is the only factor limiting growth, growth failure would result because essentially all of that substance had been utilized. This supposition was tested as follows in the case of pyridoxine:

TABLE VII

Removal of Vitamins by Bacteria from Media at Vitamin Concentrations Limiting Growth

Vitamin added	Amount per 10 cc.	Vitamin found		Per cent of original removed during growth	Turbidity of inoculated culture before centrifuging, galvanometer*
		Uninoculated	Inoculated		
	γ	γ	γ		
Pyridoxine	0	0	0		5.3
	0.5	0.48	0.50	0	28.0
	1.0	0.87	0.86	<1.2	45.0
	2.0	1 8	1.9	0	60.0
Calcium pantothenate	0	0	0		5.0
	0.03	0.05	0	100	25.0
	0.06	0.07	0	100	38.0
	0.1	0.12	0	100	50.0
Riboflavin	0	0	0		3.2
	0.1	0.095	<0.015	>84	47.0
	0.2	0.15	<0.018	>88	63.0
	0.3	0.27	<0.034	>87	75.0

* Turbidity does not increase in any case after 24 hours incubation. A reading of zero on the galvanometer indicates 100 per cent transmission; a reading of 100 is complete opacity.

To a series of tubes of base medium, pyridoxine was added in the desired quantity, with duplicate tubes at each level. The highest quantity of vitamin added in each case was just below that required by the organism for heaviest growth on the medium. One tube of each pair was then inoculated with *Streptococcus lactis*; the other was reserved as a control. Inoculated and control sets were sterilized, incubated, etc., in the same manner. After growth had ceased, the cells were centrifuged from the culture tubes. Media from all the culture tubes were then assayed for pyridoxine, and the amounts remaining after growth ceased compared to those present in the uninoculated controls. An exactly similar experiment

was carried out in which the concentration of pantothenic acid was the factor limiting growth. For this purpose the base medium already described was modified by leaving out calcium pantothenate, but adding an excess (5 γ per tube) of pyridoxine. The organism used was again *Streptococcus lactis*. Finally, a similar experiment was conducted in which riboflavin was the limiting factor. Since *Streptococcus lactis* does not require riboflavin for growth, *Lactobacillus casei* was used. The base medium was that used for riboflavin assay (11). At the end of 24 hours media from all tubes were assayed microbiologically for riboflavin. The results of these experiments are summarized in Table VII. With pantothenic acid, as with riboflavin, essentially all of the vitamin was removed from the medium before growth ceased. With pyridoxine, no detectable decrease in the concentration of the vitamin occurred as the result of growth. This can be rationalized by assuming that the organism transforms only a minute amount of the pyridoxine to the more active pseudopyridoxine, which is then utilized for growth purposes. According to this view, the necessary form of the vitamin for utilization is pseudopyridoxine, rather than pyridoxine. Why such a conversion process should cease, and growth stop, before any appreciable diminution in the vitamin content of the medium can be detected is not clear. The problem is being further investigated.

<center>DISCUSSION</center>

Evidence presented indicates conclusively that the high activity of tissue extracts in promoting growth of *Streptococcus lactis* on a pyridoxine-free medium is due to a naturally occurring, physiologically active metabolite which can be formed from pyridoxine by the animal organism, and which for this organism has many times the activity of pyridoxine itself. Indeed, the evidence suggests that pyridoxine is transformed to this substance before or during utilization.

Little can be said concerning the activity of this substance for other organisms. Several other strains of *Streptococcus lactis*, as well as certain other species of lactic acid bacteria, give similarly high assay values for pyridoxine in natural materials. Presumably the added response is due to the same substance in all cases. These responses vary from the very high values characteristic of *Streptococcus lactis* to the low values given by *Saccharomyces cerevisiae*. Such variable activity of the product with different organisms might explain why, as mentioned earlier, values obtained by the yeast growth method (6) for pyridoxine appear somewhat lower (although of the same order of magnitude) than those obtained by animal assay. If one assumes that the product has the same activity for yeast as does pyridoxine itself, one may calculate the minimum required

activity of the pure product from the ratios given in Table III. These would require that pseudopyridoxine be at least 6400 times as active as the amount of pyridoxine from which it was derived. Such a product would be detectable by its growth effect on *Streptococcus lactis* at dilutions approximating those at which biotin can be detected by this organism. This high activity would similarly explain how sufficient pseudopyridoxine could be formed from pyridoxine to permit near maximum growth of the test organism without removal of appreciable amounts of pyridoxine from the medium (Table VII).

Scudi *et al.* (16) have recently detected by chemical means an unidentified metabolite derived from pyridoxine in comparatively small amounts when animals are fed large doses of pyridoxine. Whether this substance is the same as that indicated by the above experiments is not known. Certainly the near 85 per cent of a test dose of pyridoxine which is not excreted as such by the normal adult leaves room for a variety of unrecognized metabolic products derived from this substance.

SUMMARY

1. A base medium is described which supports luxuriant growth of *Streptococcus lactis* in the presence of synthetic pyridoxine, but upon which growth completely fails in the absence of added pyridoxine or of tissue extracts.

2. Use of this medium to assess the pyridoxine content of natural extracts yields values several hundred to several thousand times as great as can be accounted for on the basis of pyridoxine actually present, as determined by other methods.

3. Experiments not published in detail showed the factor responsible for the increased activity to be very similar to pyridoxine in its behavior toward adsorbents, eluting agents, acids, and bases, and in its migration in an electric field.

4. Animal experiments showed that the active agent (provisionally called pseudopyridoxine) was greatly lowered in the tissues of pyridoxine-deficient rats, and was raised to levels which in some cases were higher than those in "normal" animals by feeding synthetic pyridoxine hydrochloride to rats maintained on the same ration. These changes were even greater than changes in the pyridoxine content of the same tissues.

5. This pseudopyridoxine effect was not due to a substance inactive by itself but sensitizing the test organism to pyridoxine already present.

6. Excretion tests on rats showed the pseudopyridoxine content of the urine paralleled the pyridoxine content of the ration, and did not parallel the pseudopyridoxine content of the ration. The same was true of concentrations in the tissues.

7. Excretion of pseudopyridoxine by a human adult following administration of a test dose of pyridoxine was greatly increased. A time lag in the excretion was present, so that the ratio of pseudopyridoxine to pyridoxine present increased as the actual amount of pyridoxine excreted fell.

8. It is concluded that pyridoxine is converted by the animal organism to a metabolite of unknown nature which possesses much greater activity for *Streptococcus lactis* than does pyridoxine itself. This metabolite is generally distributed in natural products.

9. The absorption of pyridoxine by *Streptococcus lactis* from media in which the pyridoxine concentration is the factor limiting growth does not occur to any appreciable extent, in contrast to results secured with other vitamins under similar conditions. It is suggested that this result is the consequence of a necessary conversion of pyridoxine to pseudopyridoxine prior to utilization for growth by this organism.

10. The bearing which variable activity of such a metabolite (as compared to that of pyridoxine) might have on other bioassays for pyridoxine is indicated.

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AN ACCURATE METHOD FOR THE DETERMINATION OF BLOOD UREA NITROGEN BY DIRECT NESSLERIZATION

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The multiplicity of published procedures for determining urea nitrogen in blood is an indication that the ideal method has not yet been found. That of Van Slyke and Cullen (1) is accurate but requires 3 cc. of whole blood, involves aeration, and is time-consuming. Of equal accuracy is the gasometric method of Van Slyke (2), but special apparatus and skill, not available in the ordinary clinical laboratory, are required.

Because of these factors, many simpler methods have been devised, usually at the expense of accuracy. In general they depend upon direct nesslerization, with or without the use of protective colloids, of blood filtrates in which the urea has been hydrolyzed by one means or another. While it is recognized by all that the accuracy of these simplified methods is not great, their use has been justified on the grounds that the error is not clinically significant. Of this type are the procedures proposed by Karr (3), Barrett (4), Raices (5), Wrenn (6), Taylor, Hayes, and Wells (7), Hawk and Andes (8), Naumann (9), Hoffman and Osgood (10), Ohllson (11), Sure and Wilder (12), and Hughes and Saifer (13). With reference to these methods, Folin (14) has stated:

"It is unfortunate that accurate urea determinations cannot be made by direct Nesslerization of hydrolyzed blood filtrates. The reason why this cannot be done is not so much due to the insidious turbidities, for these can be largely eliminated by the use of gum ghatti, but rather because of the presence of relatively large amounts of nitrogenous materials which have a profound effect both on the quality and on the quantity of color which the urea-ammonia gives with Nessler's reagent."

The principal sources of error in the direct nesslerization methods, therefore, are turbidity and the difference in quality of color in specimen and standard.

Turbidity is apparently due to the reduction of the complex mercuric salt of Nessler's reagent to mercurous compounds by certain constituents normally present in blood, such as creatine, creatinine, uric acid, glucose,

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etc. These reducing substances cause the development of a greenish precipitate in directly nesslerized specimens on prolonged standing.

To prevent this turbidity, Looney (15) introduced the use of gum ghatti, but we have found that the prevention is more apparent than real and depends on colloidal dispersion by the gum giving the appearance of a clear solution. Upon standing, the same greenish precipitate collects in about the same time as in the unprotected solution. Furthermore, the addition of the gum decreases the intensity of color by as much as 10 per cent (see Table I).

One of the principal complaints of those using a direct nesslerization method is that the shade of color in the unknown differs so markedly from that of the standard that when matching is done in a visual colorimeter a certain amount of guesswork is involved. The remark is frequently heard that the filtrate appears to be less red than the standard.

Spectrophotometric study should show the reason for this difference in the quality of color, but nowhere in the literature is there any reference to such work, probably because of the instability of directly nesslerized specimens (Table I).

In the method to be described, we have stabilized the color and clarity of directly nesslerized blood filtrates for a period of at least 1 to 3 hours. This permitted the establishment of a narrow band of wave-lengths over which color absorption is approximately the same for directly nesslerized specimens and for ammonium sulfate standards.

<center>EXPERIMENTAL</center>

Early in this study it became apparent that methods previously used as standards against which to compare direct nesslerization procedures would not be sufficiently accurate and reproducible for our purposes. It was necessary to have for reference a method which could be used colorimetrically to check the quantitative precision of direct nesslerization procedures, and one which would serve also as a basis for comparison of the quality of the resulting colors.

In the procedure of Van Slyke and Cullen, 3 cc. of treated whole blood are aerated and the collected ammonia determined by titration, with a negative error of 1 to 2 per cent, due to incomplete recovery of the ammonia. If this method is applied to 5 cc. of blood filtrate (0.5 cc. of blood) and the ammonia loss is of the same magnitude, the expected error will be about 10 to 12 per cent. When ammonium sulfate solutions, equivalent in nitrogen content to 0.5 cc. of blood, and blanks were run by the aeration method, the collected ammonia nesslerized, and the blank subtracted, the error was found to be exactly of this order. If the blank was ignored, closer agreement resulted. The reduction of Nessler's reagent in the blank ap-

pears to be due to substances from the rubber tubing connections which persist in spite of the most thorough cleansing.

Somewhat similar findings were obtained when the simple distillation method of Folin and Svedberg (16) was used. Here again fairly accurate figures resulted if no blank determination was made, but if the blank was deducted, standard solutions gave an error of about -10 per cent. The blank in this method was found to be due to the oil used as an antifoaming agent as well as to the rubber tubing connections. Compensating for this addition of substances reacting with Nessler's reagent is the loss which occurs during the initial stages of the distillation in which about 90 per cent of the ammonia comes over. No matter how carefully one attempts to regulate the displacement of air from the apparatus, it bubbles through the receiving acid rapidly and vigorously, carrying ammonia with it and preventing complete absorption as demonstrated by Miller (17). Only when the all-glass steam distillation micro-Kjeldahl apparatus of Kemmerer and Hallett (18) was used could the nitrogen from an ammonium sulfate solution equivalent in amount to that in 0.5 cc. of normal blood (0.05 to 0.075 mg. of N_2) be recovered with an accuracy of 1 per cent.

New Distillation Method—The reference method finally adopted, in which this apparatus is used, is based on that of Folin and Svedberg and upon the semimicro-Kjeldahl method of Redemann (19). The flask and condenser are thoroughly swept out with steam from the generator and 1 cc. of N sulfuric acid plus 10 cc. of water is placed in the distillation flask. After the hydrolyzed blood filtrate or the known ammonium sulfate solution is introduced into the distillation flask, the burner under it is started. Heating of the generator and distillation flask is continued until all air has been swept from the apparatus, indicated by cessation of bubbling through the receiver. Alkali, in this instance saturated sodium borate solution, is then added to the specimen, liberating the ammonia. Since all air has been removed, there is no possibility of loss of ammonia by dilution with it or by too vigorous bubbling through the receiving acid. Distillation is continued in the usual manner.

This method was used in all of the succeeding work. Standard solutions and blanks were distilled in duplicate with each blood filtrate and only when the results agreed within 1 per cent were they used for comparison with the corresponding direct nesslerizations.

Stabilization of Color of Nesslerized Blood Filtrates; Use of Wetting Agents—The initial clarification of nesslerized solutions obtained by Looney by the use of gum ghatti was probably due to its dispersing properties. In the hope of obtaining a more permanent dispersion, a number of wetting agents were tried. Among them were Triton NE, Santomerse No. 1, Santomerse No. 2, Avitex W, Aerosol OT, Nacconol NR, Duponol

ME, and Soricin (sodium ricinoleate). In general these agents were similar in their action to gum ghatti. Nacconol NR was definitely superior to the others. When combined with potassium persulfate their action was somewhat more efficient, but not sufficiently so to warrant inclusion in the method finally adopted.

Use of Oxidants—Since the turbidity which develops is probably due to the reduction of Nessler's reagent with the formation of insoluble mercurous compounds, the use of oxidizing agents suggested itself. Such an agent should have an oxidation-reduction potential sufficiently high to prevent reduction of the Nessler's reagent without at the same time oxidizing the ammonium salts, the other nitrogenous constituents of the blood filtrate, or the Nessler's reagent itself.

A large number of oxidizing agents, including persulfates, peroxides, hypochlorites, chlorates, and perchloric acid, were tried for this purpose. All had some effect in preventing turbidity, potassium persulfate being by far the best. However, after its addition color density progressively increased. Eventually a reddish sediment of mercuric iodide formed. This oxidation of the Nessler's reagent indicated that the oxidation-reduction potential of the potassium persulfate was too high for the purpose.

In an attempt to restrain this action of the potassium persulfate to a point where oxidation of the mercuric complex would not occur, salts of various organic acids having mild reducing properties were added. A number of compounds were tried, including lactates, citrates, maleates, phthalates, tartrates, and gluconates. The desired results were obtained with the last two, the gluconates being somewhat better than the tartrates. When potassium persulfate and potassium gluconate were added to directly nesslerized blood filtrates, the specimens remained clear, with no change in color density for a minimum period of 1 hour to a maximum of 3 hours. The color intensity then gradually increased at about 1 per cent per hour. These final reaction mixtures do, however, remain free from turbidity for days.

This stabilization of reaction may be due to the attainment of a proper oxidation-reduction potential by the addition of gluconate and persulfate or it may be due to the formation of a soluble mercury complex by the gluconate, just as tartrate in an alkaline copper solution forms a soluble complex. Potassium gluconate alone has considerable stabilizing action but eventually the Nessler's reagent is reduced and a greenish sediment forms. In all likelihood the action is a combination of both of these factors.

Analysis of Color Quality in Nesslerized Solutions—While the addition of persulfate and gluconate resulted in a clear reaction mixture which remained stable in color intensity for as long as 3 hours, it did not affect the

difference in shade of color between nesslerized hydrolyzed blood filtrates on the one hand and similarly nesslerized ammonium sulfate standards and distilled blood filtrate on the other. The increased stability of color and clarity of solution, however, did permit spectrophotometric study. From the curves so obtained and shown in Fig. 1, the reason for the difference in shade becomes apparent.
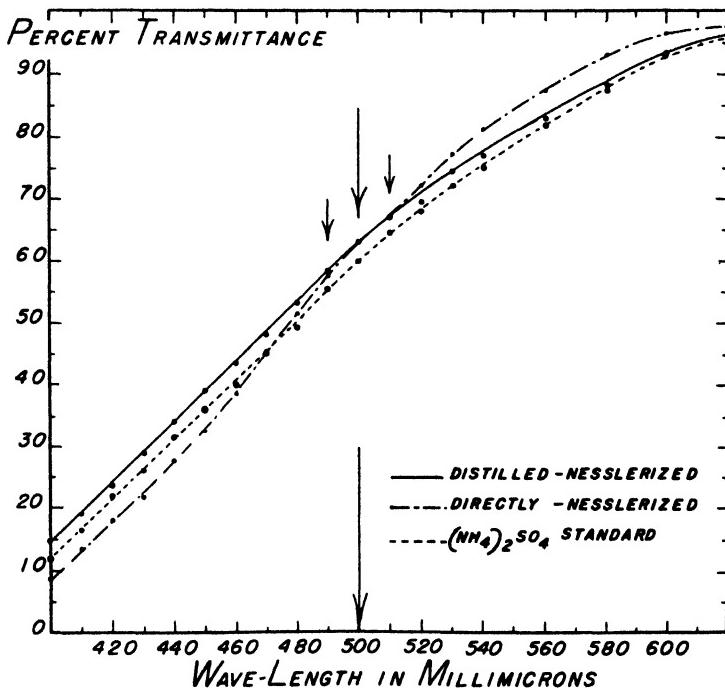
FIG. 1. Spectral transmittance of a directly nesslerized blood filtrate after urease hydrolysis compared with the same specimen in which the ammonia was separated by distillation and nesslerized. Concentration of urea N, 13.6 mg. per 100 cc. in both when compared with a standard at 500 mμ. A nesslerized ammonium sulfate solution equivalent to 15 mg. of N per 100 cc. is shown for comparison. If the ammonium sulfate solution were made exactly the same in concentration as the distilled specimen, their curves would be exactly superimposed.

By means of a Coleman photoelectric spectrophotometer, model 10-S, with fixed 5 mμ slit, the spectral transmittance of a directly nesslerized specimen was measured and compared with that of a nesslerized distillate of the same filtrate, as well as with a nesslerized ammonium sulfate solution of the same ammonia content. Repeated measurements of this kind show that transmission curves of distilled and nesslerized blood filtrates are

superimposable upon those of equimolar nesslerized ammonium sulfate standards. This is evident in Fig. 1 in which the similarity of color content in the two solutions is shown by the parallelism existing between them when their concentrations differ slightly.

This identity of curves, however, does not hold for an equivalent amount of ammonia in a directly nesslerized blood filtrate. Only between the wave-lengths of 490 and 510 mμ does the color density of the directly nesslerized specimen coincide with that of the distilled nesslerized filtrates and the standard solutions. Below this narrow band, the directly nesslerized filtrates transmit less light than do standards of equivalent ammonia concentration. Accordingly, calculations on unknowns, based on comparison with standard solutions at such wave-lengths, yield ammonia nitrogen values which are too high. Above this narrow band, the transmission of the directly nesslerized filtrates is greater than that of the standard solutions, and calculations based on the comparative color densities at these wave-lengths yield values which are too low.

Comparisons have been made by other methods at wave-lengths below 500 mμ. For example, a dominant wave-length of 440 mμ was used by Sure and Wilder and Hughes and Saifer, and one of 430 mμ by Hoffman and Osgood. That such measurements have an inherent error is evident from the graphs in Fig. 1. The curve of the standard solution represents a concentration of 15 mg. of urea N per 100 cc. of blood. By calculation against this standard as read at 500 mμ, the concentration of the unknown, distilled as well as directly nesslerized, is 13.6 mg. per 100 cc. If, however, the comparison is made at 440 mμ, the values are 13.8 and 16.76 mg., and at 430 mμ, 13.8 and 17.0 mg. per 100 cc., respectively.

An idea of the magnitude of the errors resulting when unknown directly nesslerized blood filtrates are compared with standards at wave-lengths other than 500 mμ also may be obtained by inspection of Fig. 2. The three examples given are typical of the large number of specimens varying in concentration from 6 to 75 mg. of urea N per 100 cc. that have been studied by this method.

In each case, the color densities of directly nesslerized and distilled nesslerized blood filtrates were measured at intervals over the range of the visible spectrum. The calculation of the concentration of the unknown was based upon the previously noted finding that the transmission of distilled nesslerized filtrates was identical with that of ammonia standards of equivalent concentration. Plotting the concentration of nitrogen in directly nesslerized filtrates against wave-length emphasizes the great variation in results which occurs if the comparison with standards be made at wave-lengths other than those centering at 500 mμ.

The values shown in Fig. 2, *A* were obtained with a Bausch and Lomb
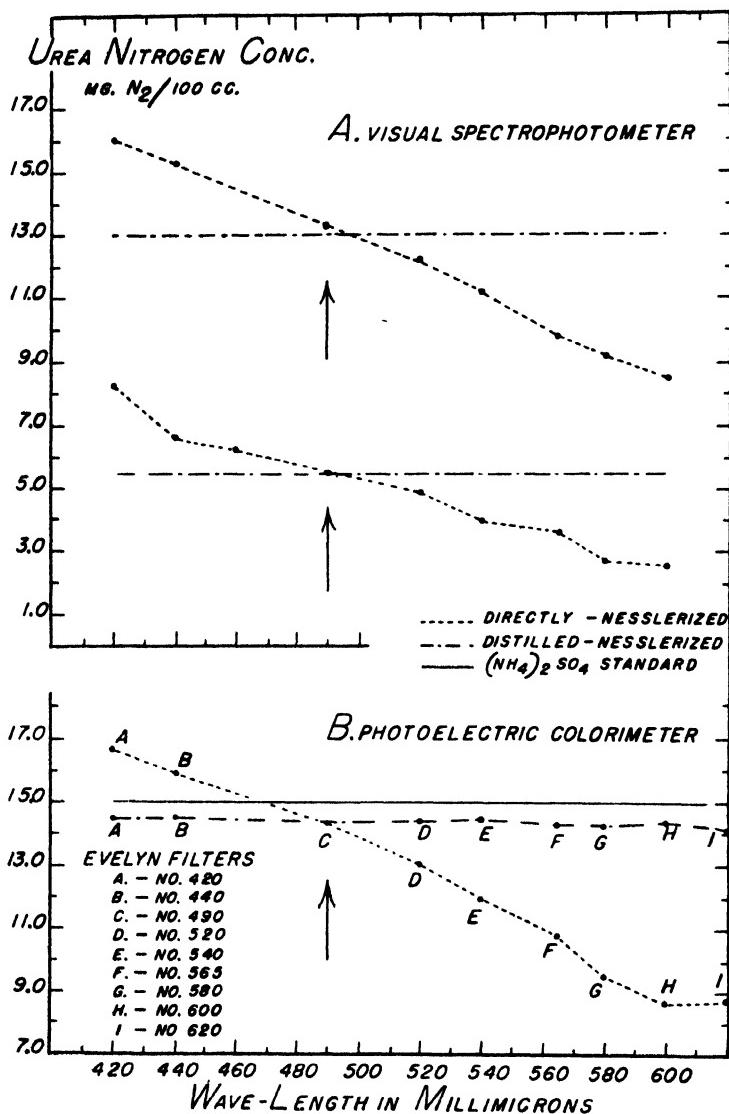
Fig. 2. *A*, the urea nitrogen concentration of two blood specimens as determined by the described method of direct nesslerization is compared with that of the same specimen in which the ammonia was separated by distillation and nesslerized. The readings were made on a Bausch and Lomb visual spectrophotometer. *B*, a comparison is made of the urea nitrogen of a single blood specimen as determined by direct nesslerization and by distillation and nesslerization. The latter is compared with an ammonium sulfate standard. The readings were made on an Evelyn photoelectric colorimeter with the filters designated.

visual spectrophotometer and those of Fig. 2, *B* with the standard Evelyn photoelectric colorimeter in which the wave bands were limited by the use of glass filters. In the latter, further verification of the relationships discussed under Fig. 1 is shown by the exact parallelism which results when the calculated concentration of a distilled nesslerized sample and that of a standard solution are plotted against wave-length.

Although the wave-length at which absorption is the same for both directly nesslerized and distilled nesslerized specimens is not always exactly at 500 mμ, it is never below 490 nor above 510 mμ.

From the foregoing presentation, it is obvious that no application of Beer's law to the color of a nesslerized blood filtrate at wave-lengths other than those centering at 500 mμ can give correct values when the standard of comparison is a known solution of ammonium sulfate. When a spectrophotometer is used, narrow wave-lengths in this region are easily obtained. When, however, a photoelectric colorimeter or a visual instrument is used, narrowness of wave band is secured by means of a proper light filter. Selection of such a filter involves a certain amount of empiricism, since monochromatic filters are neither available nor practicable. Inasmuch as filter density increases with selectivity, some sacrifice of narrowness of band must be made in order to avoid a density too great to permit sufficient light to pass.

The filter best suited in photoelectric colorimetry is that supplied with the Evelyn instrument under No. 490.[1] It is equally satisfactory with other photoelectric instruments having sufficient sensitivity to permit full scale deflection when inserted in the light path. It may be made by assembling in a suitable holder Corning Glass Works standard glasses as follows: No. 338 Noviol shade C 2 mm. thick, No. 430 dark shade blue-green 2 mm. thick, and No. 503 dark theater blue 5 mm. thick.[2]

For visual colorimetry the most suitable one was found to be Wratten gelatin filter No. 75. Visibility with this filter is fairly good and the wave-lengths transmitted below 490 mμ are about balanced by those above 500 mμ. This filter may be cemented between circular microscopic cover-slips and used in the eyepiece of the colorimeter, or between microscopic slides and used over the light source in the colorimeter base. Sand-blasting one surface of one of the slides improves diffusion. In either event the blue daylight filter must be removed, since it will alter the balance between unwanted wave-lengths.

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[1] The colorimeter and filters were manufactured by the Rubicon Company, Philadelphia.

[2] The thickness of this element may be reduced to 4 mm. for colorimeters having filter disks whose total capacity is only 8 mm. The effective transmission is not changed too greatly thereby.

Method

Reagents—

Sodium tungstate, 10 per cent aqueous solution.

Sulfuric acid, 0.66 N.

Stock standard nitrogen solution. Dissolve 7.074 gm. of ammonium sulfate, A. C. S., in 0.1 N sulfuric acid and make up to 1 liter.

Working standard. Dilute 5 cc. of stock solution to 500 cc. with 0.01 N sulfuric acid (0.015 mg. of N per cc.).

Potassium gluconate, 1 per cent aqueous solution. Store in the refrigerator. Make up a fresh solution weekly.

Potassium persulfate. A 2.5 per cent aqueous solution of the C.P. nitrogen-free salt. This solution must be prepared freshly each week and kept in the refrigerator at all times. During warm weather remove it only long enough to measure out the amount required, for decomposition is quite rapid at higher temperatures.

Urease, Squibb, double strength, powdered.

Nessler's solution. The stock solution is the original one of Folin and Wu (20), metallic mercury being used (see also (14) p. 337). This Nessler's reagent is superior to that of Koch and McMeekin (21). The final solution differs in that the alkali concentration is 5 instead of 7 per cent. Place 500 cc. of 10 per cent NaOH in a liter volumetric flask, add 150 cc. of stock Nessler's reagent, and dilute to the mark with distilled water. Allow to stand for 3 days before use to permit sedimentation of the small amount of precipitate which forms.

*Procedure—*To 5 cc. of whole oxalated blood add 35 cc. of water, then 10 to 20 mg. of the urease powder, and shake. The estimated amount may be picked up on a knife blade. Let stand at room temperature, which must not be below 20°, for 20 minutes. Next add 5 cc. of the sodium tungstate solution, mix, and then 5 cc. of the 0.66 N sulfuric acid. Shake vigorously, let stand 10 minutes, and filter through a good grade of qualitative paper. Whatman No. 2 is satisfactory. It is not advisable to use the double acid-washed papers used in quantitative analysis, since all of these have been found to give appreciable ammonia blanks, while the qualitative papers give none. After filtration has proceeded for about 10 minutes, pour the filtrate back onto the paper. The first portion of the filtrate has been found to give greater light absorption when measured with a blue filter and also higher values for urea N and non-protein N.

Place 5 cc. of filtrate in a test-tube graduated at 20 and 25 cc. In a similar tube place 5 cc. of the working nitrogen standard, containing 0.075 mg. of nitrogen, and to each add distilled water to the 20 cc. mark. Mix by shaking.

Prepare the nesslerizing solution by mixing 1 part of the gluconate and 1 part of the persulfate solutions. Pour this mixture into an equal volume of Nessler's reagent (1 gluconate + 1 persulfate + 2 Nessler's reagent). This mixture must be used within 15 minutes. To the standard and to each unknown add 4 cc. of the mixed reagent, dilute at once to the 25 cc. mark, stopper with paraffined corks, and mix by vigorous shaking. Allow to stand for 15 minutes to develop full color, then compare in a colorimeter in the usual manner, using the proper filter. Comparisons should be completed within 1 hour after nesslerization. The tubes should be kept stoppered as much of the time as possible.

If a photoelectric colorimeter is used, the proper filter is inserted, full scale deflection secured with a distilled water blank, and the standard read, then the unknowns. It is better thus to compare unknowns with a simultaneously prepared standard than it is to use a calibration curve, since the color varies greatly with temperature as shown by Pincussen (22), and with the age and lot of the reagents.

Calculation—When a visual colorimeter is used

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 15 = \text{mg. urea N per 100 cc. blood}$$

For direct reading photoelectric colorimeters with logarithmic scales the calculation is

$$\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 15 = \text{mg. urea N per 100 cc. blood}$$

With colorimeters reading per cent of transmission only, the density must be calculated. This is defined as the log of (1/transmission) or log 1 − log T. Since scale readings are in terms of per cent of transmission, the above becomes log 100 − log of reading, or 2 − log of reading = density (D). Values of D for standard and unknown are then substituted in the formula

$$\frac{D \text{ unknown}}{D \text{ standard}} \times 15 = \text{mg. urea N per 100 cc. blood}$$

Test of Method—Results obtained by this method are shown in Tables I to III. In Table I is compared the stability of color with time for two specimens treated by four methods: (1) by the Wrenn method of direct nesslerization, (2) by the Looney method with gum ghatti, (3) by the method herein described, and (4) by the new distillation method followed by nesslerization with the new mixed reagent.

Comparison of results obtained by the last two methods shows absolute agreement for a period of 90 minutes in the first specimen and for 3 hours

in the second. The apparent increase in urea nitrogen shown in the first two columns is due to increasing turbidity. After the precipitate settles out, the color is actually lighter in intensity than it was originally. The reduction in color intensity by the use of gum ghatti is also shown. All readings in Table I were made with the Evelyn colorimeter with a No. 490

TABLE I

Rate of Change in Color of Two Hydrolyzed Blood Filtrates Directly Nesslerized Compared with Distillation and Nesslerization of the Same Specimen

All values are expressed as mg. of urea N per 100 cc. of blood.

Specimen	Time	Nessler's reagent only*	Nessler's reagent + gum ghatti†	New reagent	Distilled specimen + new reagent
A	0 min.	16.7	15.0	16.7	16.7
	5 "	17.3	15.9	16.7	16.7
	10 "	17.7	16.3	16.7	16.7
	15 "	18.2	16.4	16 7	16.7
	30 "	18.5	16.7	16.7	16.7
	45 "	19.0	17.4	16.7	16.7
	60 "	19.7	18.1	16.7	16.7
	90 "	20.3	19.0	16.7	16.7
	2 hrs.	23.6	20.8	17.2	16.7
	3 "	Heavy ppt.	Heavy ppt.	19.0 (Clear)	16.7
B	0 min.	17.2	15.4	16.3	17.0
	5 "	18 15	16.6	17.15	17.1
	10 "	18.7	17.0	17.15	17.1
	15 "	19 2	17.2	17.15	17.1
	30 "	20.4	18.2	17.1	17.1
	45 "	21.4	19.7	17.1	17.1
	60 "	23 2	20.6	17.1	17.1
	90 "	26.5	22.8	17.1	17.1
	2 hrs.	Heavy ppt.	Heavy ppt.	17 1	17.1
	3 "	" "	" "	17.1	17.1
	4 "	" "	" "	17.45	17.1
	24 "	" "	" "	18.6 (Clear)	17.1

* Nesslerized only; similar to the method of Wrenn.
† Method of Looney.

filter. All distillations reported in this and in Table II were in duplicate, and blanks and standard nitrogen solutions were distilled as controls.

In Table II are shown a number of determinations on actual specimens from hospitalized patients. These determinations were made by the distillation method and the results compared with those obtained by the new method of direct nesslerization with the photoelectric colorimeter. They were compared also with the results obtained with a visual colorimeter, both with and without the aid of a Wratten filter No. 75.

Values determined by the method of direct nesslerization agree closely with those by the method requiring initial distillation of the ammonia when read on the photoelectric colorimeter. The maximum difference in the determinations listed is 2.2 per cent and the average 1.15 per cent. Results obtained when the readings were made in a visual colorimeter supplied

TABLE II

Comparative Values for Urea Nitrogen by New Distillation and New Direct Nessleri-zation Method; Readings Made by Photoelectric and Visual Colorimeters

All values in mg. per 100 cc. of whole blood.

New distillation method (Evelyn colorimeter), filter No. 490	Direct nesslerization method			
	Evelyn colorimeter, filter No. 490	Per cent deviation	Visual colorimeter	
			With Wratten filter No. 75	Without filter
13.23	13.5	+2 0	13.2	
13.25				
16.26	16.2	−0.4	16.5	
16.25				
16.7	16.7	±0	16.8	
16.7				
15.6	15.4	−1.3		
14.5	14.8	+2.1	14.7	12.0
14.6	14.8	+1.4		
13.2	13.4	+1.5	13.0	12.2
20.5	20.3	−1.0	21.0	
21.0	21.0	±0	21.5	19.0
16.0	16.3	+1.9	15.8	14.3
76.0	75.3	−0.9	77.4	
43.7	44.2	+1.1		
17.2	17.0	−1.2		
18.0	18.1	+0.5		
9.1	9.3	+2.2	9.4	
5.52*	5.56	+0.9	5.8	No reading possible
5.51				

* Case of toxemia of pregnancy; no reading possible by visual colorimeter without filter because of difference in color quality.

with the Wratten filter are also in reasonable agreement. Without the filter there is considerable error.

The one instance of extremely low urea nitrogen concentration was a case of suspected toxemia of pregnancy. It was absolutely impossible to do a determination on this specimen by any of the older methods, for upon the addition of Nessler's reagent a very heavy precipitate immediately formed.

As a final check on the method, experiments were conducted to determine the extent of recovery of urea added to blood samples, the urea concentration of which had already been determined. These data are recorded in Table III. Recoveries ranged from 97.2 to 98.6 per cent.

Precautions—A few precautions must be observed in using this method. The range over which good proportionality between standard and unknown holds is about 2-fold. With a standard equal to 15 mg. of urea nitrogen per 100 cc., the concentration of the unknowns should be not less than 8 nor more than 30 mg. per 100 cc. For ranges beyond these limits a new standard must be prepared, or the filtrate diluted before nesslerization.

The clearest blood filtrates are produced by the original method of Folin and Wu. It is superior to any of the modifications, particularly those in which the acid is added before the tungstate or in which blood is added directly to N/12 acid. Filtrates prepared in such manner show an appreciable absorption of light when measured in a photoelectric colorimeter with

TABLE III

Recovery of Urea Nitrogen Added to Blood

All values in mg. per 100 cc. of whole blood.

Urea N originally present	Amount added	Amount recovered	Per cent recovered
12.8	5.0	17.5	98.0
15.3	15.0	29.6	97.7
10.3	25.0	34.8	98.6
15.6	45.0	58.9	97.2

a blue filter. This absorption varies from 8 to 12 per cent as compared with 2 per cent by the original Folin-Wu filtrate. The zinc hydroxide filtrate of Somogyi (23) offers no advantage.

It is essential that the first portion of the filtrate be poured back onto the filter, for this first portion not only shows less transparency when measured with a blue filter but also yields higher values for non-protein and urea nitrogen.

Although urease solutions are frequently used, and their activity may be preserved indefinitely by the use of glycerol, such solutions nevertheless tend to develop ammonia blanks, even when relatively fresh, owing to the action of a secondary enzyme as shown by Howell (24). This difficulty is avoided by using a dry preparation. The double strength urease powder prepared by Squibb has a sufficiently high enzymatic activity and is entirely free of ammonia and other non-protein nitrogen. These criteria were not met by the other commerical preparations tested.

SUMMARY

A simple accurate method of determining urea nitrogen in blood by direct nesslerization is' described. No elaborate apparatus nor unusual reagents are required.

In the method potassium persulfate is used to prevent the reduction of Nessler's reagent and potassium gluconate to restrain the oxidizing action of the persulfate, form a soluble mercury complex, or to do both. By these means a color is produced which is stable for a minimum of 1 hour. The final reaction mixture remains clear and sparkling for days.

Spectrophotometric study of the color of the final nesslerized solution indicates that color comparisons should be made between the wave-lengths of 490 and 510 mμ with 500 mμ the optimum point. Appropriate filters for photoelectric and visual colorimeters are described.

As a basis for accurate comparison a new distillation procedure is outlined which gives accurate results with the amount of urea normally present in 0.5 cc. of whole blood.

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CANINE CYSTINURIA. THE EFFECT OF FEEDING CYSTINE, CYSTEINE, AND METHIONINE AT DIFFERENT DIETARY PROTEIN LEVELS*

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The question whether cystinuria, long under study in man, also affects animals was answered affirmatively in 1935 by Morris, Green, Denkel, and Brand (1) who reported the removal of a cystine stone from an Irish terrier. Shortly thereafter the son of a male litter mate of the same dog was found to be cystinuric (2). By breeding the younger cystinuric dog with related females (non-cystinuric) Brand *et al.* (3–5) obtained more than 300 descendants of which twelve were definitely cystinuric, all males. Through the courtesy of Dr. Brand two of these cystinuric Irish terriers, designated as Dogs 32-T and 38-U, were placed at our disposal.

As reported by Brand, Cahill, and Kassell (6) the reaction of the fresh urine of these dogs was negative by the Sullivan direct method. After the urine had been aged for several days, the Sullivan reaction became positive. Brand *et al.* (2) believe that the reaction is negative owing to the presence in the urine of an unknown reducing substance which interferes with the color development. If the cystine in the urine was precipitated with cuprous chloride according to the method of Rossouw and Wilken-Jorden (7) and the copper removed from the washed precipitate by hydrogen sulfide, the filtrate, after concentration, contained free cystine determinable by the Sullivan method. The question as to why the Sullivan method is negative in the freshly voided urine will be considered in detail in a later paper. However, we have evidence (Howard and Sullivan (8)) that certain cysteine complexes of the thiazolidine type which are negative in the Sullivan reaction are quantitatively opened by precipitation with cuprous chloride and then react positively.

It is the conclusion of all workers that in human cystinuria the urinary cystine increases with increased protein intake. Brand, Cahill, and Harris (9) have postulated that the error of metabolism in the case of cystinuria is concerned with the ingested methionine and cysteine and not with the ingested cystine. Lewis, Brown, and White (10) also found that both methionine and cysteine stimulated the excretion of cystine in a human cystinuric but to a lesser degree than found by Brand *et al.* (9) and re-

ported that the stimulation was more marked upon a lower protein diet than upon a high protein diet. Hess and Sullivan (11) in a study of two cases of human cystinuria found a stimulation of cystine excretion following the ingestion of both methionine and cysteine with one cystinuric but not with the other.

In their early study of canine cystinuria Brand, Cahill, and Kassell (6) confined their investigation to the determination of cystine, creatinine, total nitrogen, and sulfur distribution. Later (5) Kassell, Brand, and Cahill reported that with increasing protein (casein) intake the cystine excretion increases. In our investigation we have studied not only the effect of various levels of dietary protein upon the cystine excretion in two cystinuric dogs but also the effect of the administration of methionine, cysteine hydrochloride, and cystine upon the cystine excretion at the various levels of protein intake.

<div align="center">EXPERIMENTAL</div>

The diet was that recommended by Dr. Brand and had the following percentage composition: casein[1] 25, sucrose 45, salt mixture[2] 1.5, bone ash[3] 3.5, yeast[4] 5, and lard 20. Daily, 5 cc. of cod liver oil were added to the diet of each dog. When the level of casein in the diet was changed, the sucrose was varied inversely. The diet was prepared fresh once a week. The amino acids fed, l-cystine, l-methionine, and cysteine hydrochloride, were analytically pure. The cysteine hydrochloride was prepared from the same lot of cystine as was used in the feeding experiments. The l-methionine was isolated from casein. The weighed amount of each amino acid was intimately mixed with the diet each day. Each dog was fed approximately 150 gm. of diet per day.

As communicated to us by Dr. Brand in an extension of the data of Brand et al. (6) Dog 4 was bred with a non-cystinuric female, Dog 2, to give a cystinuric male, Dog 4-19, and a non-cystinuric female, Dog 4-22. Dog 4 bred with female Dog 3 gave female Dog 9-31. The latter bred with Dog 4-19 gave Dog 32-T. Dog 4-22 bred with Dog 4-19 gave female Dog 17-A which bred to Dog 4-19 gave the cystinuric Dog 38-U. Dog 32-T was born April 25, 1939, and Dog 38-U was born May 11, 1939. The dogs were kept in separate metabolism cages. The urine, collected daily, was

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[1] Casein Company of America, edible casein No. 453 (sulfate-free).

[2] The salt mixture prepared was as follows: ferric citrate 9 gm., potassium iodide 2.5 gm., potassium chloride 35 gm., calcium phosphate ($CaHPO_4 \cdot 2H_2O$) 39 gm., monobasic potassium phosphate 61 gm., magnesium citrate 163 gm., sodium chloride 190 gm. To this mixture are added 100 to 200 mg. each of copper tartrate, cobalt chloride, manganese phosphate, and zinc carbonate.

[3] Eimer and Amend, fine, Cupel, Michigan (sulfate 0.24 per cent).

[4] Fleischmann's dried yeast, 7.56 per cent N, 0.37 per cent S.

analyzed immediately for cystine, total nitrogen, total sulfur, total sulfate sulfur, and inorganic sulfate sulfur. Dog 32-T weighed 7 kilos and Dog 38-U weighed 9 kilos.

TABLE I

Average Daily Urinary Excretions during Control Periods on Various Protein Levels

Urine	Dog No.	Protein level			
		5 per cent	10 per cent	25 per cent	50 per cent
		gm.	*gm.*	*gm.*	*gm.*
Cystine	32-T	0.008	0.039	0.082	0.144
	38-U	0.008	0.042	0.082	0.144
Nitrogen	32-T	0.96	1.98	5.16	9.33
	38-U	0.95	2.04	5.21	9.39
Total S	32-T	0.096	0.138	0.249	0.462
	38-U	0.095	0.147	0.247	0.482
Neutral S	32-T	0.050	0.061	0.111	0.196
	38-U	0.045	0.066	0.119	0.233

TABLE II

Total Urinary Excretion for 4 Day Period during Ingestion of Amino Acids upon Various Protein Levels

Urine	Dog No.	Protein level									
		5 per cent	10 per cent	25 per cent	50 per cent	5 per cent	10 per cent	25 per cent	50 per cent	5 per cent	25 per cent
		2.0 gm. methionine				2.3 gm. cysteine hydrochloride				2 gm. cystine	
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Cystine	32-T	0.104	0.289	0.293	0.537	0.084	0.233	0.323	0.545	0.033	0.335
	38-U	0.137	0.355	0.405	0.560	0.092	0.381	0.370	0.518	0.038	0.333
Nitrogen	32-T	4.22	8.44	24.0	39.7	4.24	8.78	23.7	41.5	3.83	22.2
	38-U	4.05	8.41	23.2	39.9	4.51	9.40	23.3	43.3	3.94	21.4
Total S	32-T	0.627	0.776	1.166	2.253	0.819	1.068	1.558	2.193	0.807	1.406
	38-U	0.626	0.799	1.187	2.416	0.851	1.023	1.559	2.180	0.881	1.263
Neutral S	32-T	0.284	0.328	0.497	0.891	0.427	0.373	0.667	1.119	0.371	0.615
	38-U	0.319	0.291	0.576	0.883	0.381	0.339	0.626	1.073	0.367	0.590

The urine as collected contained no cystine sediment and, in fact, gave no reaction for free cystine by the Sullivan method and recourse was had to precipitation with cuprous chloride. An aliquot of the urine (25 cc. for the lower casein levels and 10 cc. for the 50 per cent level) was precipitated with cuprous chloride (6), the copper was removed from the complex with hydrogen sulfide, and the filtrate was concentrated to the original volume of the aliquot of the urine. During the concentration the cysteine

was oxidized to cystine and in the Sullivan method cystine was employed as the standard.

The casein in the diet was varied in content from 5 to 50 per cent in four different levels, 5, 10, 25, and 50 per cent. Table I gives the average daily excretion of the principal substances determined in the urines of both dogs. The average value in each case is based upon a considerable number of determinations made both before and after the feeding of the various amino acids. Since the amount of diet furnished to each dog and eaten was the same each day, the values upon which the averages are based are, in all cases, close together.

Table II presents the data on the effect of feeding methionine, cysteine hydrochloride, and cystine on the same urinary constituents. Of the amount of each amino acid indicated, one-half was fed daily for 2 successive days and the urine was collected for these 2 days and also for the next 2 days to insure complete elimination of any cystine produced by the ingestion of the compound administered.

<center>DISCUSSION</center>

The two dogs do not differ greatly in their response to the different levels of casein in the diet. The greatest variation is in the neutral sulfur output on the 50 per cent casein diet (Table I). The effect of the different protein levels in the diet upon the excretion of urinary cystine is marked. In agreement with the findings of Brand *et al.* (5) there is a sharp increase in urinary cystine following each increase in the level of dietary protein. It may be noted that the increase in cystine output is proportionally greater than the increase in protein intake, especially upon the lower levels of protein. The excretion of nitrogen increases directly as the dietary protein is increased but such a proportionality does not occur in the neutral sulfur output.

The ingestion of methionine and cysteine hydrochloride by the dogs causes the excretion of far more extra cystine upon the 5 and the 10 per cent casein levels than upon the 25 and the 50 per cent levels. In fact on the higher levels of protein intake these amino acids cause little if any increase in cystine excretion over that on the basal diet. The actual increase in cystine output following the administration of any amino acid can be calculated by subtracting from the amount excreted in the 4 day period, as given in Table II, 4 times the daily average for the corresponding casein level as given in Table I. The results are given in Table III together with the actual percentage increase of the cystine excreted over the control period for each amino acid fed. In general Dog 38-U gives a more marked response to the amino acid fed than does Dog 32-T, particularly upon the lower casein levels. However, the trend with both dogs is the same.

Both the methionine and the cysteine hydrochloride exert their most marked effect upon the cystine excretion when the casein is fed at a 5 per cent level. Increasing the casein level to 10 per cent and feeding either methionine or cysteine hydrochloride produces an increased output of cystine but the increase is far less than on the 5 per cent casein level. When the casein level is further increased to 25 or 50 per cent, the increase or decrease in extra cystine excreted is well within the experimental error of the methods. Whether the absolute amount of cystine excreted is considered or the percentage increase over the normal, it is evident that the two lower levels of dietary casein permit methionine and cysteine hydro-chloride to bring about the excretion of extra cystine in the urine, while the

TABLE III

Urinary Excretion of Extra Cystine Following Ingestion of Amino Acids
(4 Day Period)

Protein level	Dog No.	Methionine	Cysteine	Cystine
per cent		*gm.*	*gm.*	*gm.*
5	32-T	0.072 (225)*	0.052 (163)*	0.001 (3)*
	38-U	0.105 (328)	0.060 (188)	0.006 (19)
10	32-T	0.133 (85)	0.077 (49)	
	38-U	0.187 (111)	0.213 (126)	
25	32-T	−0.035 (−11)	−0.005 (−2)	0.011 (3)
	38-U	0.077 (23)	0.042 (13)	0.009 (2)
50	32-T	−0.039 (−6)	−0.031 (−5)	
	38-U	−0.016 (−3)	−0.058 (−10)	

* The figures in parentheses are the percentage increase in cystine excreted above the control.

two higher levels do not. This finding with cystinuric dogs is in agreement with the finding of Lewis, Brown, and White (10) with human cystinuria that the excretion of cystine is stimulated by methionine and cysteine more on a higher protein intake than on a lower. In no case did the feeding of cystine have any influence upon the cystine output.

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CARBOHYDRATE CHARACTERIZATION

III. THE IDENTIFICATION OF HEXURONIC OR SACCHARIC ACIDS AS BENZIMIDAZOLE DERIVATIVES*

By ROLLAND LOHMAR, ROBERT J. DIMLER, STANFORD MOORE, AND KARL PAUL LINK

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The first paper of this series by Moore and Link (1) gives a procedure for the characterization of certain aldo-monosaccharides as benzimidazole derivatives. The method has now been extended to include the identification of hexuronic acids as the dibenzimidazole derivatives of the corresponding saccharic acids. The procedure gives readily characterized derivatives of *d*-glucuronic, *d*-mannuronic, and *d*-galacturonic acids under identical conditions of treatment.

The characterization of aldoses (1, 2) involves three general steps: (*a*) liberation of aldoses from polysaccharides or glycosides by acid hydrolysis, (*b*) the oxidation of the aldoses to the aldonic acids by hypoiodite in methanol, (*c*) the condensation of the aldonic acids with *o*-phenylenediamine to form the aldo-benzimidazoles. Similarly, the basis of the proposed method for hexuronic acids involves liberation of the aldehyde sugar acid, its oxidation to the saccharic acid, and subsequent benzimidazole formation.

Hexuronic acid   A saccharo-dibenzimidazole
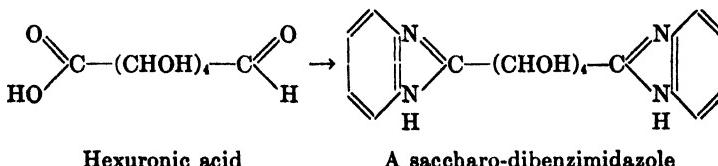
The application to the hexuronic acids, however, necessitates modifications of the first two steps. The sensitivity of uronic acids to acid hydrolysis may cause extensive decomposition in the first step. In general the familiar simultaneous hydrolysis-oxidation by hydrobromic acid-bromine has proved the most useful method for converting polyuronides to the saccharic acids. On the other hand, if the free acid has

been obtained, it is preferable to carry out the oxidation to the saccharic acid by the bromine-barium benzoate method of Hudson and Isbell (3). While the hypoiodite oxidation used for the aldoses is applicable to galacturonic acid, it fails to give satisfactory results with glucuronic and mannuronic acids.

With unknown samples giving a positive color test for uronic acid, two types of treatment may be considered. One procedure is the use of hydrobromic acid-bromine (or bromine-barium benzoate) and identification of both aldoses and hexuronic acids as the benzimidazole derivatives in the one series of operations. The fractionation of the mixture of derivatives is facilitated by the lower solubility of the saccharo-dibenzimidazoles in comparison with the aldo-benzimidazoles. An alternate procedure is first to characterize the aldoses (1).[1] On a separate sample the hydrobromic acid-bromine technique may be applied in search for hexuronic acids, with the advantage of previous knowledge of the aldo derivatives to be expected.

The hydrolysis-oxidation with hydrobromic acid-bromine produces a solution in which the total mineral acid present is in excess of the amount desired for the o-phenylenediamine condensation. Excess of hydrobromic acid can be a cause of low benzimidazole yields. Complete removal of hydrobromic acid with silver carbonate makes it possible to establish the optimum acid concentration in the hydrochloric-phosphoric acid condensation mixture. Partial removal of hydrobromic acid by concentration under reduced pressure may be used but the yields are less reproducible.

The dibasic acids formed by the oxidation of the hexuronic acids are condensed with o-phenylenediamine under conditions similar to those employed for the aldonic acids (1). The optimum molar ratio of diamine to carboxyl group is 1.3:1, with 1 mole of hydrochloric and 1 mole of phosphoric acid per mole of diamine for condensation. The yields are in general satisfactory, although not necessarily maximum, when the treatment of unknowns involves deviation from these proportions. Diethylene glycol is used to keep the mixture fluid during the 2 hour condensation at 135°.[2] The picrates are prepared as directed for the aldo-benzimidazoles (1). The hydrochlorides of the saccharo-dibenzimidazoles are less soluble than those of the aldo-benzimidazoles and can be readily obtained from aqueous acid solution.

The three dibasic acids, d-saccharic, d-mannosaccharic, and mucic, derived from d-glucuronic, d-mannuronic, and d-galacturonic acids are

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[1] The presence of uronic acid decomposition products (from acid hydrolysis and potassium hypoiodite-methanol oxidation) does not have a tendency to interfere with aldose identification.

[2] If aldonic acid components of a mixture are to be characterized at the same time, it is preferable to omit the diethylene glycol.

characterized by the four constants given for each benzimidazole derivative in Table I. The advantages in ease of isolation and characterization which the saccharo-dibenzimidazoles possess over hydrazine derivatives (4) or salts of sugar acids are similar to those enumerated for the aldobenzimidazoles. In the case of mucic acid, of course, the advantage in isolation is less marked than in the other instances, but the properties of the benzimidazole are preferable for characterization.

Since this procedure is a characterization of the dibasic acids resulting from the oxidation of hexuronic acids, its interpretation is subject to the general stereochemical limitations of saccharic acid isolation. Differentiation between d-galacturonic and l-galacturonic, or between d-glucuronic

TABLE I

Saccharo-dibenzimidazole Derivatives

Hexuronic acid	Dibenzimidazole		Hydro-chloride, m.p.*	Picrate, m.p.*
	M.p.*	$[\alpha]_D^{25}$		
	°C.	*degrees*	°C.	°C.
d-Glucuronic	238	+60.3†	257–258	211
d-Mannuronic	250	−1.3‡	256–257	241
d-Galacturonic . . .	298	0.0†	318	250

* All melting points represent melting with decomposition, uncorrected, in a Thiele apparatus below 270° and in an aluminum block above 270°, with a temperature rise of 2° per minute.

† Rotations in 5 per cent citric acid (aqueous) with c = 2 (approximate).

‡ Aqueous solution of hydrochloride, c = 2 (solubility insufficient in citric acid). The tetraacetate, from pyridine-acetic anhydride, gives $[\alpha]_D^{25} = -11.9°$ (c, 2; CHCl₃), m.p. 225–226°. Acetyl calculated, 32.95; found, 31.90, 32.00 (Kuhn-Roth).

and l-guluronic acids, is not accomplished. In work with natural products the derivatives may permit a preliminary characterization in favor of d-galacturonic or d-glucuronic acid and indicate experiments for final identification of the isomer. d-Mannosaccharo-dibenzimidazole is completely characteristic for d-mannuronic acid. The possibility of the preexistence of the saccharic acids as such in natural products gives rise to the problem of benzimidazole preparation prior to oxidation and methods of this type are under investigation.

EXPERIMENTAL

Condensation from Saccharic Acids—Per 5 mM (1.04 gm.) of a saccharic acid (or, for example, 0.87 gm. of mannosaccharic dilactone) add 1.40 gm. (13 mM) of o-phenylenediamine, 1.1 cc. (13 mM) of concentrated HCl, 1.1 cc. (13 mM) of syrupy H₃PO₄, and 4 cc. of diethylene glycol in a 6 inch

test-tube. Warm the mixture on a steam bath until solution is realized, add a boiling chip, and place the tube in an oil bath at 135° ± 5° for 2 hours. Dissolve the syrupy residue in 5 to 10 cc. of water, add a small amount of carbon, and filter through asbestos. To the warm filtrate add concentrated NH_4OH until the solution is alkaline. Crystallization is completed by cooling the solution. Filter the crystals and wash with water, acetone, and ether. The yield of the dibenzimidazole is about 1.6 gm. or 90 per cent of theory from saccharic, mannosaccharic, or mucic acid (analytical data in Table II).

TABLE II

Analyses of Saccharo-dibenzimidazoles

Procedure	Dibenzimidazole		Nitrogen	
			Calculated	Found
			per cent	*per cent*
Dumas	Free base	*d*-Saccharo-	15.82	15.86
		d-Mannosaccharo-	15.82	15.75
		Mucic acid	15.82	15.80
Kjeldahl	Picrate	*d*-Saccharo-	17.25	17.16
		d-Mannosaccharo-	17.25	17.34
		Mucic acid	17.25	17.48

TABLE III

Analyses of Saccharo-dibenzimidazole Dihydrochlorides

Dibenzimidazole $(HCl)_2$	Carbon (calculated, 50.57), found	Hydrogen (calculated, 4.72), found	Chlorine (calculated, 16.60); (Carius) found
	per cent	*per cent*	*per cent*
d-Saccharo-	50.28	4.40	16.45
d-Mannosaccharo-	50.43	4.50	16.50
Mucic acid....	50.35	4.55	16.29

Recrystallization—The benzimidazoles of the saccharic acids are less soluble in water and organic solvents than the benzimidazoles of the aldonic acids. In general, for recrystallization suspend the derivative in hot water, bring into solution by the addition of dilute HCl, decolorize with carbon, and add a slight excess of NH_4OH to the hot filtrate. A volume of about 20 cc. per 0.1 gm. of benzimidazole is satisfactory. *d*-Saccharo-dibenzimidazole may be recrystallized from a large volume of ethanol.

Preparation of Hydrochlorides—Dissolve 0.1 gm. of the benzimidazole in 4 cc. of hot N HCl. After a brief period of cooling, the hydrochloride crystallizes out in long needles. Wash with acetone, then ether. The yield is almost quantitative. Analytical data are shown in Table III.

Treatment of Free Uronic Acids—Oxidize 0.25 gm. of glucuronic acid by the bromine-barium benzoate procedure proposed by Hudson and Isbell (3) for the aldoses. In this case it is necessary to add enough barium benzoate to neutralize the carboxyl group of the uronic acid as well as the HBr produced in the oxidation. After removal of the inorganic ions and benzoic acid, condense the syrup containing the saccharic acid with 0.35 gm. of o-phenylenediamine in the presence of 0.8 cc. of 4 N HCl, 0.8 cc. of 4 M H_3PO_4, and 1 cc. of diethylene glycol for 2 hours at 135°. Take up in water, decolorize, and make the hot filtrate alkaline with NH_4OH. The yield of dibenzimidazole is about 0.37 gm. per 0.25 gm. of uronic acid taken, or 80 per cent of theory.

Treatment of Glycuronides—Reflux 1 gm. of borneol glucuronide for 2 hours in 25 cc. of a solution of N HBr containing 1 cc. of bromine. Aerate the solution to remove the bromine and then filter. For removal of excess HBr, concentrate the solution under reduced pressure at 40° to a volume of 2 cc. Add 25 cc. of ethanol and again concentrate the solution to a small volume. Do this twice. Condense the thin syrup in the usual manner with 0.85 gm. of o-phenylenediamine, 0.6 cc. of syrupy H_3PO_4, and 2 cc. of diethylene glycol. The yield of d-saccharo-dibenzimidazole is 48 per cent of theory.

Decompose 1 mM (0.551 gm.) of barium methylmannuronide with N sulfuric acid. An excess of the sulfuric acid should be avoided. Carry out the oxidative hydrolysis as outlined above. Remove the excess bromine by aeration and the hydrobromic acid with silver carbonate. Add H_2S to remove soluble silver salts. Concentrate the solution under reduced pressure at 40° to about 4 cc. For condensation, use 0.28 gm. of o-phenylenediamine, 0.65 cc. of 4 N HCl, 0.65 cc. of 4 M H_3PO_4, and 1 cc. of diethylene glycol. The yield of d-mannosaccharo-dibenzimidazole is 41 per cent of theory. Parallel experiments with removal of the hydrobromic acid by concentration under reduced pressure give yields of 30 per cent.

Pectic acid and alginic acid may also be subjected to this procedure. With these substances, a prolonged (about 24 hours) period of hydrolysis-oxidation is necessary. From a 1 gm. sample of pectic acid, 0.55 gm. of mucic acid dibenzimidazole is obtained. From 1 gm. of alginic acid, 0.36 gm. of d-mannosaccharo-dibenzimidazole is obtained. With poly-uronides which hydrolyze with difficulty, the hydrobromic acid-bromine procedure involves appreciable destruction and the benzimidazole yields are low.

SUMMARY

A method is described for the identification of the naturally occurring hexuronic acids, *d*-glucuronic, *d*-mannuronic, and *d*-galacturonic, as

dibenzimidazole derivatives of the corresponding saccharic acids. The applicability of the procedure has been tested on hexuronic acids, glycuronides, and polyuronides.

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THE CHARACTERIZATION OF LACTIC ACID AS THE BENZIMIDAZOLE DERIVATIVE*

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In view of the suitability of the benzimidazole derivative for the characterization of lactic acid, the constants on the optically active derivatives should be recorded (Table I). The racemic lacto-benzimidazole (2-(α-hydroxyethyl)-benzimidazole) has been prepared by several investigators (1–5) but only the specific rotation has been reported for the optically active derivatives (6). Furthermore the usefulness of the preparation of

TABLE I

Physical Constants of Lacto-benzimidazole ($C_9H_{10}ON_2$)

Optical form	M.p.	Hydrochloride, m.p.	Specific rotation (c, 4; EtOH)
	°C.	°C.	*degrees*
D or *L*	175–177	213–215	D +33.4
			L −33.4
DL	179–181	211–213	0.0

the hydrochloride as a secondary derivative with a characteristic melting point has not been indicated.

Lactic acid present in aqueous solution, after preliminary separation from any major quantities of interfering substances, may be converted to lacto-benzimidazole either by the general procedure outlined for the quantitative determination of the isomer ratio in commercial samples (6) or by the method given in the application of benzimidazole derivatives to carbohydrate characterization (7). The former procedure, based on the isolation of the crystalline silver salt, will usually be found more convenient because of the essentially complete isolation of the derivative, the additional analytical constants which may be obtained readily on the silver salt (Ag 40.1 and N 10.4), and the convenience of determination of the specific rotation.

The free base may be obtained from the alcohol solution used for rotation measurement by concentration to dryness and recrystallization of the lacto-benzimidazole from hot water-alcohol (14:1). The melting points of mixtures of the active and racemic derivatives may be 2–5° below the melting points of the pure L ("sarco") and DL compounds. The hydrochlorides (Table II) may be prepared by the procedure used in carbohydrate characterization (7). The addition of ether is effective when the hydro-

TABLE II

Representative Analyses of Lacto-benzimidazoles

Benzimidazole, $C_9H_{10}ON_2$	Silver (calculated, 40.11); (Volhard) found	Nitrogen (Dumas)		Chlorine (calculated, 17.85); (Carius) found
		Calculated	Found	
	per cent	*per cent*	*per cent*	*per cent*
Ag salt of D form	40.11	10.40	10.0	
	40.00		10.2	
" " " DL "	40.05	10.40	10 0	
	40.05		10.2	
D form (free base)		17.28	17.00	
			16.80	
Hydrochloride of D form		14.13	14.05	17.50
			14.00	17.46
" " DL "		14.13	14.00	17.48
			13.99	17.48

chloride does not crystallize readily from the acetone-alcohol mixture. The picrate is not readily obtained.

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LETTERS TO THE EDITORS

THE FORMATION OF CYSTEINE FROM *ll*-S-(β-AMINO-β-CAR-BOXYETHYL)HOMOCYSTEINE BY LIVER TISSUE

Sirs:

In a recent communication[1] it was reported that the unsymmetrical thio ether, *ll*-S-(β-amino-β-carboxyethyl)homocysteine could serve in lieu of cystine in the diet of rats for the support of growth. It was inferred that the compound was converted to cystine. We now wish to report direct evidence that liver tissue is capable of cleaving this thio ether to form cysteine.

Slices of rat liver (1.8 gm., dry basis) were shaken for $2\frac{1}{2}$ hours with 50 mg. of the thio ether in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 at 38° with Ringer's solution as modified by Krebs.[2] Trichloroacetic acid was then added to give a concentration of 5 per cent. The presence of 7.8 mg. of cystine in the neutralized filtrate was indicated by the Folin-Marenzi method as modified by Kassell and Brand.[3] The cystine was precipitated as the cuprous mercaptide and after regeneration of cystine the Sullivan method showed the presence of 6.0 mg. of cystine. In a series of runs the cleavage averaged 20 per cent of the theoretical value at $2\frac{1}{2}$ hours and 30 per cent at 5 hours. Anaerobically the cleavage was somewhat higher and production of H_2S was observed. Control runs without thio ether gave no appreciable Sullivan test. When the Ringer's solution with suspended liver slices was heated to boiling, no cleavage of the thio ether was obtained. Muscle and kidney tissue were inactive.

Saline extracts of fresh rat livers were active in cleaving the thio ether. 5 cc. of extract representing 2.5 gm. of fresh liver gave an average cleavage of 30 per cent of thio ether in $2\frac{1}{2}$ hours at 38° in phosphate buffer at pH 7.5 under N_2. An appreciable production of H_2S was observed. The H_2S-free solution gave strongly positive tests for cysteine. For quantitative analysis the cysteine was converted to cystine by aeration. Since the Folin-Marenzi and Sullivan values agreed quite closely in most of the experiments, it would appear questionable that appreciable amounts of homocysteine were formed, a conclusion which agrees with that drawn from the growth studies with the compound.[1] To remove any doubt that cysteine had been formed by the action of the extract, the isolation of cystine was attempted. The combined aerated filtrates from several runs containing approximately 40 mg. of cystine as indicated by colorimetric

[1] du Vigneaud, V., Brown, G. B., and Chandler, J. P., *J. Biol. Chem.*, **143**, 59 (1942).
[2] Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
[3] Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938).

analysis were used.   17 mg. of cystine were isolated in crystalline form. The liver extract heated for 10 minutes at 100° was found to be inactive. The addition of NaCN inhibited H₂S production but the cleavage of the thio ether still occurred, thus indicating that the thio ether enzyme is distinct from the enzyme studied by Fromageot[4] and by Smythe[5] which forms H₂S from cysteine.

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[4] Laskowski, M., and Fromageot, C., *J. Biol. Chem.*, **140**, 663 (1941).
[5] Smythe, C. V., *J. Biol. Chem.*, **142**, 387 (1942).

CYSTINE, TYROSINE, AND ARGININE - CONTENT OF HIGH POTENCY PRESSOR AND OXYTOCIC PITUITARY HORMONES*

Sirs:

Using the separated principles obtained by the base exchange method which we have briefly reported[1] and subjecting the two fractions to further purification, we have been able to obtain amorphous powders of consistently high biological activity. The details of purification will be reported in the near future; we desire, however, to call attention to the hydrolysis products of these two principles, since the preparations we have investigated have higher potency than any as yet recorded in the literature. The oxytocic preparation was a non-deliquescent white powder which dissolved readily in water to form a colorless solution. Upon standing in an evacuated desiccator at $-3°$ it slowly discolored, with some loss in biological activity. It assayed 700 units per mg. by the method of Coon.[2] The pressor principle was a similar product which assayed 450 units per mg. in the anesthetized cat. The oxytocic preparation had less than 20 units and the pressor principle less than 40 oxytocic units per mg.

10 mg. of each of these preparations were hydrolyzed according to the method of Gurin and Clarke[3] and on the hydrolysate cystine was determined by the Sullivan method,[4] tyrosine by the method of Arnow,[5] and arginine by a slight modification of the method of Dumazert and Poggi;[6] readings were made with an Evelyn photoelectric colorimeter and appropriate filters. The following values were obtained for the oxytocic preparation, cystine 18.3, tyrosine 14.2, arginine < 0.8 per cent; for the pressor principle, cystine 19.0, tyrosine 11.9, arginine 12.3 per cent. The total sulfur of the oxytocic preparation was 5.59 per cent,[7] which is in agreement with the value for the cystine; unfortunately lack of material at this time prevented an analysis for sulfur on the pressor principle. Recently Irving and du Vigneaud in an excellent review[8] have summarized the present

[1] Potts, A. M., and Gallagher, T. F., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.,* **140**, p. ciii (1941).

[2] Coon, J. M., *Arch. internat. pharmacod.,* **62**, 79 (1939).

[3] Gurin, S., and Clarke, H. T., *J. Biol. Chem.,* **107**, 395 (1934).

[4] Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.,* **117**, 423 (1937).

[5] Arnow, L. E., *J. Biol. Chem.,* **118**, 531 (1937).

[6] Dumazert, C., and Poggi, R., *Bull. Soc. chim. biol.,* **21**, 1381 (1939).

[7] Analysis by Dr. T. S. Ma of the Department of Chemistry, University of Chicago.

[8] Irving, G. W., Jr., and du Vigneaud, V., New York Academy of Sciences, Conference on protein hormones of the pituitary body, January 9, 10 (1942).

status of the hormones of the posterior lobe of the pituitary gland and our values should be compared with those of other investigators recorded there.

The strikingly low content of arginine in the oxytocic preparation is in contrast with the significant amount of the amino acid in the pressor substance. We believe that this difference can account for the fact that the pressor principle behaves as a more basic ampholyte (isoelectric point 10.8 according to Cohn, Irving, and du Vigneaud[9]) and is more strongly adsorbed on permutit than is the oxytocic preparation (isoelectric point 8.5[8]). We propose to study the usefulness of this difference in arginine content as a chemical method for following the purification of the pressor hormone.

It is interesting likewise to note that the cystine and tyrosine values for the oxytocic fraction are in very good agreement with a minimum molecular weight of approximately 1300, assuming but a single molecule of each amino acid (1311 from cystine and 1274 from tyrosine). The pressor hormone gives a similar value, although here the agreement is not as good, which is in accord with the lower degree of purification for this fraction. It should be emphasized that these products were not crystalline; we believe, however, the possibility that a chemical method of assay may be used at least on highly purified products warrants presentation at this time.

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[9] Cohn, M., Irving, G. W., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **137**, 635 (1941)·

DIMERIC *dl*-GLYCERALDEHYDE-1,3-DIPHOSPHATE

Sirs:

The synthesis of glyceraldehyde-3-phosphoric acid was reported 10 years ago.[1] Recent work of Negelein and Brömel[2] and Warburg and Christian[3] has made it appear very likely that a glyceraldehyde-1,3-diphosphate is an intermediate of sugar metabolism. This compound, Substance A, has now been synthesized in a dimeric form (see the formula).

```
HO
  \
   O=P·O·CH₂
  /      |    O
HO       |   / \
        HC    HC·O·P=O
         |    |      \
HO       |    |       OH
  \      |    |
   O=P·O·CH   CH
  /        \ / |      OH
HO          O  |     /
              CH₂·O·P=O
                     \
                      OH
```
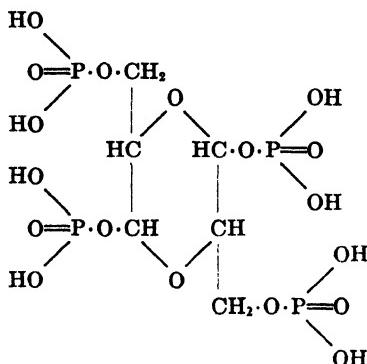
Dimeric *dl*-glyceraldehyde was phosphorylated by treatment in pyridine with diphenyl phosphorus oxychloride (method of Brigl and Müller[4]). The octaphenyl ester of Substance A was obtained, needles, m.p. 108–109°. Found, C 58.2, H 4.6, P 11.5. Catalytic hydrogenation in methanol with platinum oxide and hydrogen at room temperature resulted in a smooth cleavage of the protecting phenol groups, yielding Substance A. The crystalline acid barium and calcium salts were prepared by adding barium and calcium chloride and subsequently ethanol. Both salts crystallized in small well defined needles.

$C_6H_{12}O_{18}P_4Ba_2$ + 2H₂O.   Calculated.   C 8.93, H 1.50, P 15.36, Ba 34.05
                     Found.   " 8.82, " 2.01, " 15.4, " 32.8
$C_6H_{12}O_{18}P_4Ca_2$ + 2H₂O.   Calculated.   " 11.77, " 1.98, " 20.24, Ca 13.09
                     Found.   " 11.57, " 2.7, " 19.4, " 12.7

In contrast to the calcium salt of glyceraldehyde-3-phosphoric acid, the barium and calcium salts of Substance A do not form methylglyoxal spon-

[1] Fischer, H. O. L., and Baer, E., *Ber. chem. Ges.*, **65**, 337, 1040 (1932).
[2] Negelein, E., and Brömel, H., *Biochem. Z.*, **301**, 135 (1939).
[3] Warburg, O., and Christian, N., *Biochem. Z.*, **303**, 40 (1939).
[4] Brigl, P., and Müller, H., *Ber. chem. Ges.*, **72**, 2121 (1939).

taneously on standing. Moreover, the new ester is stable against moderate concentrations of alkali (pH 9.6) in the cold. However, in hot acid solution in the presence of 2,4-dinitrophenylhydrazine, a quantitative yield of methylglyoxal dinitrophenylosazone is precipitated.

The new ester contains, like the Cori ester (glucose-1-phosphate), phosphoric acid residues linked to "glycosidic" hydroxyl and, in addition, phosphoric acid residues bound to primary alcoholic hydroxyl. A difference in the ease of hydrolysis of the two types of linkage is apparent from the shape of the hydrolysis curves.

The percentage hydrolysis of Substance A in 0.1 N hydrochloric acid at 100° is shown in the following figures.

Time, min...	5	10	15	20	30	60	120
Inorganic P, %...	27	47	59	62	71	82	100

The hydrolysis curve obtained from these figures closely resembles that of triose monophosphates, up to 50 per cent hydrolysis. Thereafter, the hydrolysis is markedly slower. This behavior in acid solution may serve to differentiate the new ester from the triose monophosphates. The ester is readily hydrolyzed also by phosphatases. Details of all experimental studies will be published later.

Investigations on the probable rôle of this compound as an intermediate of sugar metabolism are in progress.

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CARBON DIOXIDE ASSIMILATION IN CELL-FREE LIVER EXTRACTS*

Sirs:

Although the assimilation of CO_2 by a variety of plant and animal cells is a well recognized phenomenon, a similar reaction in cell-free extracts has not been described. We should like to report, therefore, the preparation of cell-free extracts of pigeon liver which assimilate 10 to 50 per cent of the radioactivity added as $C^{11}O_2$. The experimental conditions are similar to those previously described.[1] These extracts are made by grinding minced pigeon liver with sand and an equal volume of ice-cold 0.1 M phosphate buffer, pH 7.4, and centrifuging. The cloudy, cell-free supernatant fluid retains its activity for several days at 0°. There is a slow loss of activity on dialysis. In the undialyzed extracts there is an initial rapid uptake of CO_2 (measured as per cent of total C^{11} present) which reaches a maximum in about 10 minutes and is followed by a gradual decrease. Apparently both assimilating and CO_2-releasing reactions occur in such preparations.

Distilled water or phosphate buffer extracts of acetone-dried pigeon liver also show active CO_2 assimilation. With these extracts there is an increase in the amount of CO_2 assimilated with time and it seems probable that the reactions leading to the release of assimilated CO_2 are either inhibited or absent. In a typical experiment, 4 cc. of water extract (1 gm. of acetone powder + 8 cc. of water incubated 5 minutes at 40° and centrifuged) were added to 4 cc. of Ca-free bicarbonate-Krebs' solution equilibrated with 5 per cent CO_2, 95 per cent oxygen in the presence of 1 cc. of 0.1 M pyruvate, 0.25 cc. of 0.1 M succinate, and $C^{11}O_2$. The mixture was incubated at 40° in a 50 cc. vessel in an atmosphere of 5 per cent CO_2, 95 per cent O_2. Examination of metaphosphoric acid filtrates at intervals showed that 6, 8, 21, and 24 per cent of the C^{11} was fixed as organic carbon at 10, 20, 60, and 120 minutes, respectively. In the absence of added pyruvate and succinate only about 0.5 per cent C^{11} is fixed in 20 minutes. The water extract shows only slight loss of activity on standing in the ice box 24 hours. There is greater, but not complete, loss after 24 hours dialysis against distilled water.

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[1] Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).

VELOCITY OF COMBINATION OF ANTIBODY WITH SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS*

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Aggregation of staphylococci by immune serum was observed by Eagle (1) to take place at room temperature in 15 seconds but much more slowly at 4°. It was shown in this laboratory by means of quantitative chemical analysis (2) that combination of Type I pneumococci with homologous antibody in horse serum, without regard to aggregation, was 80 per cent complete in a sample removed after 5 minutes at 0.5°, although the reaction period was lengthened to a possible 10 to 15 minutes by the necessity of centrifugation (also in the cold[1]). Similar experimental difficulties prevented Follensby and Hooker (3) from establishing at much less than 8 minutes the initial rapid reaction between diphtheria toxin and antitoxin postulated earlier by Pappenheimer and Robinson (4). A more exact calorimetric timing of the reaction between hemocyanin and antibody in horse serum (5) showed that heat evolution was 80 per cent complete in 2 minutes after mixing.

In the meantime application had been made in this laboratory of the principle of competitive reactions which has rendered such service in the kinetic study of inorganic and organic chemical reactions. It was found (6) that addition of rabbit anti-egg albumin to a suitable mixture of egg albumin (Ea) and horse anti-Ea serum at 0° within about 20 seconds produced no greater reversal of the soluble reaction product of the immune horse serum reaction than did addition of rabbit anti-Ea after a week. Since addition of Ea to a mixture of horse and rabbit anti-Ea sera had shown that both forward reactions proceeded at similar rates, this was interpreted to mean that combination of Ea and horse anti-Ea to form soluble compounds was essentially complete within 20 seconds at 0°.

Additional studies of this type are now reported for certain homologous and cross-reactions of horse antibody and pneumococcus polysaccharides. The cross-reaction between Types III and VIII pneumococci was chosen

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* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital and is to be submitted by Manfred Mayer in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

[1] In an International Equipment Company refrigerated centrifuge.

because it has been studied extensively by the quantitative precipitin technique (7, 8).

Antipneumococcus Type VIII horse sera contain antibody fractions which react with Type III specific polysaccharide (S-III) as well as with the homologous Type VIII specific polysaccharide (S-VIII). Experimental conditions were so chosen that S-III and S-VIII competed for the cross-reactive antibody molecules in the resulting precipitin reaction. The outcome of this competition could then be determined by analysis of the supernatants for S-III and S-VIII.

EXPERIMENTAL

To 1.0 or 2.0 ml. of antipneumococcus Type VIII serum No. H-909 (1938),[2] diluted with the desired amount of saline, 1.0 ml. portions of solutions of S-III and S-VIII were added with thorough mixing at measured intervals in the order stated (Table I) at 0–10°. The same proportions of serum to S-III and S-VIII were used in every instance. Approximately one-half of the experiments was done with 1.0 ml. of serum, 0.076 mg. of S-III, and 0.150 mg. of S-VIII, while 2.0 ml. of serum, 0.152 mg. of S-III, and 0.300 mg. of S-VIII were used in the remainder in order to provide larger amounts of supernatants for analysis. Doubling the experimental quantities did not affect the outcome of the competition (Experiments 12 and 13). A unit volume of 3.5 ml. was chosen for a single quantity run and 7.0 ml. for double quantities except in several earlier experiments with double amounts at 4.5 ml. (Experiments 3, 8, 11, 18) in which volume was not a decisive factor. Two experiments (Nos. 4 and 17) were carried out at 14 ml. with double quantities in order to test the effect of dilution. Polysaccharide additions were made rapidly from calibrated 1 ml. tuberculin syringes. With the cooperation of several persons it was possible to make the minimum interval between the polysaccharide additions as short as 3 seconds. A further decrease would have involved appreciable errors in timing with the technique used. All reaction mixtures flocculated before centrifugation was started. At the end of the reaction time given in Table I the tubes were centrifuged in the cold. Aliquot portions of supernatant were analyzed for S-III by addition to an accurately measured volume of a calibrated sample of Type III antipneumococcus horse serum No. H-792[2] from which the antibody reactive with S-VIII had been removed (9). Only a few of the supernatants were analyzed for S-VIII (with S-III-absorbed Type VIII antipneumococcus horse serum), since the effects observed were more clearly reflected by the variation in S-III than

in S-VIII. The latter reacts with all of the antibody, while S-III combines only with the cross-reactive antibody fractions which are competed for by the two polysaccharides.

TABLE I

Combination of Antibody with Specific Polysaccharides of Pneumococcus

Experiment No.	Antipneumococcus VIII serum volume	S-III added	S-VIII added	Total volume	Order of polysaccharide addition to antiserum	Interval between polysaccharide additions	Reaction time between addition of second polysaccharide and centrifugation	Total reaction time*	S-III in supernatant†	S-III in ppt. (by difference)†
	ml.	*mg.*	*mg.*	*ml.*		*sec.*	*min.*	*min.*	*mg.*	*mg.*
1	1.0	0.076	0.150	3.5	Simultaneous	0	5	10	0.049	0.027
2	1.0	0.076	0.150	3.5	"	0	60	65	0.060	0.016
3	2.0	0.152	0.300	4.5	"	0	1000 (Ca.)	1000 (Ca.)	0.075	0.001
4	2.0	0.152	0.300	14	"	0	5	10	0.057	0.019
5	1.0	0.076	0.150	3.5	S-VIII first	3	5	10	0.079	0.000
6	2.0	0.152	0.300	7.0	" "	7	5	10	0.073	0.003
7	1.0	0.076	0.150	3.5	" "	20	5	10	0.072	0.004
8	2.0	0.152	0.300	4.5	" "	10	1000 (Ca.)	1000 (Ca.)	0.077	0.000
9	1.0	0.076		3.5	S-III only		5	10	0.007	0.069
10	1.0	0.076		2.5	" "		45	50	0.009	0.067
11	2.0	0.152		4.5	" "		2500 (Ca.)	2500 (Ca.)	0.009	0.067
12	1 0	0.076	0.150	3.5	" first	3	5	10	0.033‡	0.043
13	2.0	0.152	0.300	7.0	" "	3	5	10	0.034	0.042
14	2.0	0.152	0.300	7.0	" "	12	5	10	0 016	0.060
15	2.0	0.152	0.300	7.0	" "	60	5	11	0.011	0.065
16	1.0	0.076	0.150	3.5	" "	180	2	10	0.009	0.067
17	2.0	0.152	0.300	14	" "	3	5	10	0.047	0.029
18	2.0	0.152	0.300	4.5	" "	10	1000 (Ca.)	1000 (Ca.)	0.075	0 001
19	1.0	0.076	0.150	3.5	" "	180	1000 (")	1000 (")	0.040	0.036
20	1.0	0.076	0.150	3.5	" "	90,000 (Ca.)	1000 (")	2500 (")	0.020‡	0.056

* This includes the first 5 minutes of centrifugation, since inspection at that time showed that the precipitate had settled. Centrifugation was, however, continued for another half hour in order to clear the supernatant thoroughly for the subsequent analysis.

† Calculated to 1.0 ml. of serum.

‡ Single analysis only.

0.150 mg. of S-VIII (a slight excess), when added alone, precipitated 1.00 mg. of antibody N from 1.0 ml. of Type VIII antipneumococcus horse serum No. H-909. 31 per cent of the total antibody N was precipitable in the cross-reaction by 0.076 mg. of S-III (a slight excess).

S-III and S-VIII were prepared according to the methods previously described (10, 7). Samples for the preparation of quantitative solutions

were dried to constant weight *in vacuo* over P_2O_5 at room temperature. Quantitative precipitin analyses were carried out as described (11) and nitrogen analyses by a modification of the Pregl micro-Kjeldahl method.

DISCUSSION

It has become customary in this laboratory to consider specific immune precipitation (12) and the closely related specific agglutination of bacteria (2) as due to the combination of antigen and antibody which are multivalent with respect to each other; that is, molecules of antigen may become attached to molecules of antibody through one or more of several linkages on either type of molecule. The initially formed antigen-antibody complex may, by virtue of the plurality of reactive groupings, combine with additional antigen or antibody molecules or with already formed antigen-antibody compounds until gigantic aggregates are built up and separate from solution, or, in the case of bacteria, clump together and settle. This conception of the reaction mechanism appears to account most satisfactorily for the main body of knowledge regarding this immune reaction, and possesses the additional advantages of resting on and being in accord with modern concepts of protein and carbohydrate structure and being susceptible to expression in quantitative form in most instances in which sufficiently precise data are available. Not only has a somewhat similar view been proposed by Marrack (13), but there is an increasing tendency to view the industrially important polymers in much the same light (14–16), since these also arise by combination of polyfunctional substances. In accord with such a mechanism the speed of this series of reactions should diminish as the interacting immune aggregates grow larger (*cf.* (17)).

The process of immune combination has been shown to be reversible (18, 17, 4). Under suitable conditions aggregates may dissociate into uncombined antigen and antibody molecules, but the dissociation tendency of most immune reactions is relatively small. Since dissociation of an aggregate involves the breaking of many immune linkages the over-all speed of reversal should depend, at least in part, on the extent of aggregation, for the larger the immune aggregate the more linkages must be broken in its dissociation.

The data now assembled may be interpreted with the aid of these considerations and without new assumptions.

Simultaneous addition of S-III and S-VIII to anti-S-VIII and centrifugation after 5 minutes (Experiment 1) resulted in precipitation of 0.027 mg. of S-III, or 39 per cent of the total with which the antibody is capable of combining in the absence of S-VIII. The forward cross-reaction therefore takes place with a velocity roughly of the same order as that of the homologous S-VIII-anti-S-VIII reaction. When the simultaneously

added polysaccharides were permitted to react for 60 minutes before centrifugation (Experiment 2) only 0.016 mg. of S-III (23 per cent) remained in combination. After 17 hours (Experiment 3) only 0.001 mg. of S-III (1.5 per cent) was left in the precipitate. A slow liberation of S-III from combination evidently occurred. Experiments 9, 10, and 11 showed that, in the absence of S-VIII, S-III was not eliminated from the precipitate.

If one considers a single antibody molecule (A) in contact with S-III and S-VIII, it may take part in the following initial reactions which are represented as reversible, but with the equilibrium point far to the right.

(1) $\qquad$ A + S-III $\rightleftharpoons$ A·S-III
(2) $\qquad$ A + S-VIII $\rightleftharpoons$ A·S-VIII
(3) $\qquad$ A·S-III + S-VIII $\rightleftharpoons$ S-III·A·S-VIII
(4) $\qquad$ A·S-VIII + S-III $\rightleftharpoons$ S-III·A·S-VIII, etc.
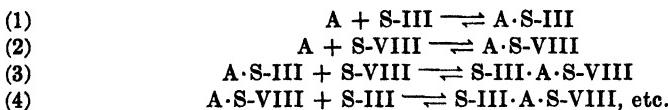
Antibody molecules or reactive antibody sites set free by reversal, as from right to left, may enter into combination with either polysaccharide. However, the slow liberation of S-III observed would indicate that the course of the reaction in the case of anti-S-VIII tends toward combination with S-VIII. In terms of the reversible equilibrium reactions formulated such a shift indicates a greater dissociation tendency of the heterologous compound. This explanation had already been given (7) for the presence of both antigen and antibody in equivalence zone supernatants in this cross-reaction.

When S-VIII, the homologous antigen, was given a head start over S-III (Experiments 5 to 8), no S-III was found in the precipitate even when the interval between additions was as short as 3 seconds. If one omits, for the moment, consideration of the possibility of appreciable reversal in so short a period, the conclusion seems justified that combination of S-VIII and anti-S-VIII was essentially complete in less than 3 seconds even at the low temperature used.

It is less easy to interpret the results of Experiments 12 to 20 in which S-III was given a head start over S-VIII. When S-VIII was added to the serum only 3 seconds after S-III (Experiments 12 and 13), 0.043 and 0.042 mg. respectively of S-III were found in the precipitates compared with 0.069 mg. of S-III when no S-VIII was introduced (Experiment 9). In Experiments 14, 15, and 16 in which S-VIII was added to the serum 12 seconds, 1 minute, and 3 minutes after S-III, 0.060, 0.065, and 0.067 mg., respectively, of S-III were found in the precipitate. At first sight this would appear to indicate that approximately 1 minute was required for complete reaction of S-III with anti-S-VIII. Alternatively, since S-VIII reacted in less than 3 seconds and since it was found that S-III and S-VIII reacted with velocities of roughly the same order, one may assume that S-III also reacted completely within 3 seconds in Experiments 12 and 13 but

that liberation of S-III through reversal took place to a measurable extent during the 10 minutes after addition of S-VIII. Thus, instead of 0.069 mg. of S-III, as in Experiment 9, only 0.042 and 0.043 mg. were found in the precipitates, corresponding to a liberation during 10 minutes of about 40 per cent of the weight of S-III originally bound. In a similar calculation applied to Experiment 1 account is taken of the fact that only one-third of the antibody present is cross-reactive. Therefore of the 0.150 mg. of S-VIII added, roughly 0.050 mg. competed with 0.076 mg. of S-III in Experiment 1, since roughly 0.100 mg. of the S-VIII would be expected to react with the two-thirds of the total antibody which does not cross-react. If the molecular weights of the two polysaccharides and their initial reaction velocities are similar, two-fifths of the cross-reactive antibody should enter into combination with S-VIII and three-fifths with S-III in proportion to these quantities of polysaccharides. Then 3/5 × 0.069 or 0.041 mg. of S-III should be bound. Instead 0.027 mg. of S-III was found in the precipitate, corresponding to a release through reversal of 34 per cent of the amount calculated as combined (0.041 mg.), in good agreement with the percentage of reversal found above. These admittedly crude calculations show that about one-third of the weight of S-III originally in combination may be released during 10 minutes. On this basis it is possible to estimate the effect of S-III liberation on the outcome of Experiments 5 to 8 in which S-VIII was added before S-III. Since the largest quantity of S-III found in the precipitate was 0.004 mg., no more than 0.006 mg. of S-III (0.004 × 3/2) could have reacted, or less than 9 per cent of the weight of S-III with which the cross-reactive antibody fraction can combine (0.069 mg.). Therefore at least 91 per cent of the cross-reactive antibody reacted with S-VIII in less than 3 seconds. Since the cross-reactive antibody is indistinguishable from the rest of the antibody as far as S-VIII is concerned (7, 8), this reaction velocity may be considered to apply to the homologous reaction as a whole.

In Experiments 1, 2, and 3, in which the polysaccharides were added simultaneously, it was shown that liberation of S-III could be completed in less than 17 hours. Release of all S-III was also demonstrated within the same period in Experiment 18 in which S-VIII was added to the antiserum 10 seconds after S-III. It is evident from Experiments 12 to 20 that the longer the interval before addition of S-VIII the slower is the reversal. This is as expected, for as stated before, the over-all speed of S-III liberation would depend at least in part on the state of aggregation at the time of S-VIII addition. Longer head starts permit aggregation and possibly other molecular rearrangements to proceed far enough to prevent liberation even over prolonged periods. For example, S-III-anti-S-VIII which had been allowed to aggregate for 2 days in the cold and which had then been centrifuged and washed was shaken mechanically in

the ice box for 4 weeks with a solution of S-VIII and a drop of toluene. No release of S-III was observed.

Experiments 4 and 17 were run in double volume in order to obtain further evidence on the mechanism of S-III reversal. While dilution would be expected to retard both forward reactions to about the same extent, the resulting delay in aggregation should accelerate S-III release through reversal. Less S-III was indeed found in these precipitates than in those of the corresponding experiments, Nos. 1, 12, and 13, in more concentrated solution.

The rapid initial S-III release (elimination of one-third of the bound S-III in 10 minutes) may be related to the existence in the cross-reactive antibody of a fraction which behaves toward S-III like univalent antibody (8). This fraction, which is multivalent toward the homologous S-VIII and which comprises approximately 30 to 50 per cent of the cross-reactive antibody, may release combined S-III more rapidly than the remaining polyvalent cross-reactive antibody fractions. It might be expected that retention of S-III in many of the precipitates would be accompanied by a decrease in the amount of combined S-VIII. In Experiments 8, 13, 14, and 15 the supernatants were accordingly analyzed for S-VIII as previously indicated. 0.002, 0.004, 0.005, and 0.006 mg. were found, respectively, showing that 0.148, 0.146, 0.145, and 0.144 mg. of S-VIII were present in the precipitates. It is thus seen that in the presence of only a very slight excess of S-VIII (0.150 mg. total) the quantity of S-VIII combined with the antibody is not significantly affected by the extent of combination with S-III and that the liberation of S-III is not due to exchange with additional S-VIII in solution. However, Experiments 9, 10, and 11 show that the S-III is not liberated unless S-VIII is present. An explanation of the difficulty may be sought in the demonstrated multivalence of specific polysaccharides (19, 7). This renders it possible for a molecule of S-VIII already in combination to react still further with antibody by virtue of uncombined reactive sites or groupings and thus effectively to displace the more easily dissociated cross-reacting polysaccharide, S-III. If this interpretation is correct, it would indicate that quite extensive intramolecular rearrangements may take place before specific polysaccharide-antibody precipitates attain their final gel-like form. It would certainly appear inadequate to attribute a dynamic process of this nature to a mere physical aggregation due to the presence of salt, as was the older view.

SUMMARY

Chemical combination of S-III and anti-S-VIII and of S-VIII and anti-S-VIII is at least 90 per cent complete in less than 3 seconds at 0°. Subsequent polymerization leading to the formation of insoluble aggregates

takes place with progressively diminishing velocity. In the presence of homologous polysaccharide the cross-reacting S-III may be liberated from combination with antibody. The velocity of this effect also diminishes as aggregation progresses. Complete elimination of S-III within a finite time occurs only when S-VIII is added during the earlier stages of the reaction.

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THE CHEMICAL DETERMINATION OF THIAMINE*

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A number of methods, chemical (1–5) as well as microbiological (6–9), have been employed for the determination of thiamine. While the former require the cumbersome adsorption of thiamine, the latter, although ideally suited for microdeterminations, are too time-consuming.

A reagent which on reaction with thiamine would produce a precipitate or a specific color in aqueous solution, which in turn could be extracted by the use of a selective solvent, would be highly desirable. The elimination of adsorption and other means of purification would greatly improve a chemical assay for thiamine. The search for such a reagent has been, it is believed, successful to the extent that the test developed can be applied quantitatively to solutions containing 3 to 6 γ of thiamine per 5 cc.

EXPERIMENTAL

Of the numerous aromatic amines investigated, ethyl p-aminobenzoate ($NH_2 \cdot C_6H_4 \cdot COOC_2H_5$), was found to produce a pink to red color when added in diazotized form to solutions of thiamine. The trichloroacetate ($Cl_3C \cdot COOHNH_2 \cdot C_6H_4 \cdot COOC_2H_5$) of this amine is fairly stable in alcoholic solutions for at least 2 months. It was also found that this latter compound would give more accurate and sensitive results than the hydrochloride. It is for this reason that the trichloroacetate of the ester is preferred.

Preparation of Trichloroacetate of Ethyl p-Aminobenzoate—16.5 gm. (0.1 mole) of ethyl p-aminobenzoate are dissolved in about 250 cc. of approximately 2 per cent HCl. To this solution are added 16.3 gm. (0.1 mole) of trichloroacetic acid in aqueous solution. The mixture is stirred for a few minutes and then filtered. The precipitate on the filter is recrystallized from 95 per cent alcohol. M.p. 158.5–160° (corrected). Analysis, Cl calculated, 3.47 per cent; found, 3.55, 3.46 per cent.

Reagents—

1. A 0.5 per cent solution of the trichloroacetate of ethyl p-aminoben-

zoate in 95 per cent alcohol. This solution was found to be stable for at least 2 months.

2. A 0.5 per cent solution of sodium nitrite in about 85 to 90 per cent alcohol, which is prepared as follows: Dissolve 1 gm. of the material in about 10 to 13 cc. of distilled water and add sufficient 95 per cent alcohol to make 200 cc. When kept cool, this reagent remains stable for about 2 months.

3. 1 N sodium hydroxide (approximate).

4. Acetic acid, approximately 10 per cent.

5. Freshly prepared sodium bisulfite solution, about 1 per cent.

6. Isoamyl alcohol.

7. Sodium sulfate, anhydrous.

All the reagents used were of "reagent quality" with the exception of the ethyl *p*-aminobenzoate, which was Eastman Kodak Company's Red Label.

Directions for the Test—The quantity of the solution to be tested depends somewhat on the concentration of the thiamine. For convenience in reading the Pulfrich photometer, it is suggested that the amounts of the vitamin used for each test range between 3 and 10 γ.

To an appropriate amount of the solution containing the thiamine, add distilled water to bring the volume to about 25 cc. The 10 per cent acetic acid is then added to bring the pH of the solution to about 5 (nitrazine paper). Next add 5 cc. of a diazo solution, prepared by mixing equal parts of Reagents 1 and 2. The reaction mixture is allowed to stand for about 2 minutes and then made alkaline with the 1 N sodium hydroxide (nitrazine paper). Again let the mixture stand for an additional 2 minutes during which time the maximum intensity of the color will be developed. 5 cc. of isoamyl alcohol are added next and the mixture is thoroughly shaken. The layers are allowed to separate and the alcoholic layer dried over anhydrous sodium sulfate. If the mixture becomes emulsified, it is centrifuged in order to speed the separation.

Standard Curve—The data given in Table I represent the reproducibility of the method on solutions of known strength. Fig. 1 shows that the readings on the Pulfrich photometer increase proportionally with a corresponding increase in the concentration of the thiamine. A standard curve can be constructed on semilogarithmic graph paper, from which the concentration of the vitamin B_1 can be read directly.

Specificity of Reaction—In order to ascertain whether or not the reagent would produce colors with other biologically important compounds, it was tested on the following substances: casein, egg albumin, urea, uric acid, acetone, creatinine, glutathione, cystine, nucleic acid, guanidine, tyrosine, amino acid mixture (Swift and Company, No. 37989), adrenalin, atropine,

homatropine, quinine, pentobarbital, phenobarbital, nicotine, histamine, sulfanilamide, insulin, lactose, dextrose, fructose, saccharin, caffeine, methyl alcohol, capryl alcohol, hydroquinone, phloroglucinol, phenol,

TABLE I

Reproducibility of Method for Determination of Thiamine

Units per 5 cc. (20 determinations each)	Variations in deviations of average, units vitamin B₁	
1	1	1
2	2	2
5	5	5
10	9	10 5
15	14	16
20	19	21
30	28	32
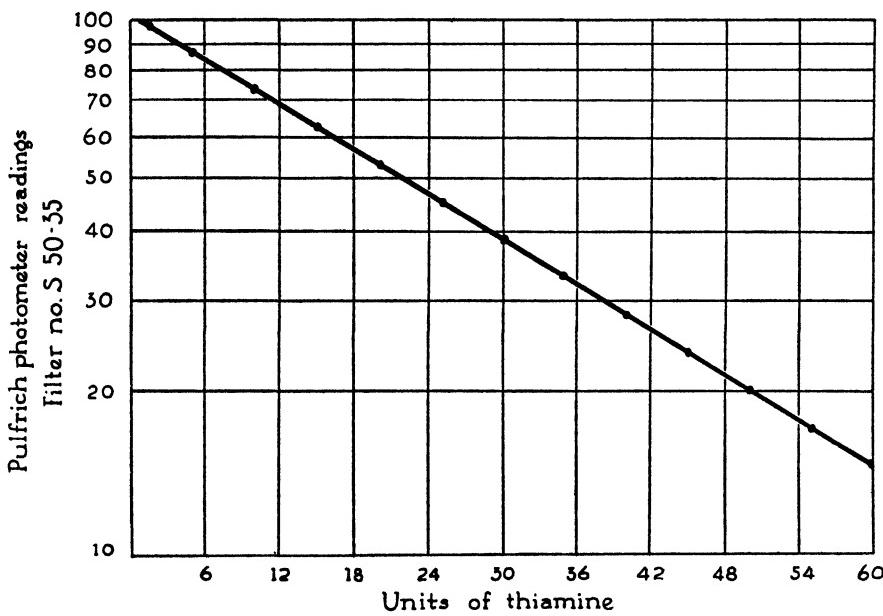
FIG. 1. Standard reference curve for thiamine

resorcinol, ethyl acetate, aspirin, methenamine, sulfathiazole, salicylic acid, and some of the vitamins.

Most of these compounds do not react to give a color which can be extracted with the isoamyl alcohol, or if a color is produced it is yellow, which fades in a short time.

Adrenalin gave a distinct red color which was soluble in the isoamyl

alcohol but it had completely disappeared after 8 to 12 hours, while the color produced by the thiamine is stable for at least 1 month. Tests were conducted on solutions containing both the adrenalin and the thiamine. The reaction mixture was allowed to stand overnight before the alcoholic layer was separated. At that time only the color due to the thiamine was present. The values obtained were identical with those obtained by the control.

Resorcinol, phloroglucinol, and phenol, in a more concentrated solution, gave orange-brown colors which faded after 14 hours. In concentrations less than 1:1000 no interference was noticeable or could be removed by shaking the isoamyl alcohol after the separation with about 2 cc. of distilled water.

In an attempt to find out which part of the thiamine molecule is responsible for the color formation on reacting with the reagent, the following intermediates used in the synthesis of thiamine were investigated: 2-methyl-5-bromomethyl-6-aminopyrimidine dihydrobromide (Merck), 2-methyl-5-ethoxymethyl-6-aminopyrimidine (Merck), and 4-methyl-5-β-hydroxyethylthiazole (Merck). 1 mg. portions of the pyrimidine intermediates did not produce a color under the conditions of the test. With the thiazole fraction, when the same quantity was used per test, a pink-red color was obtained similar to the one produced by thiamine itself, but the formation of the color was not as rapid and not as intense as in the case of the vitamin.

The following vitamins were investigated for any possible interference with the test.

1. Vitamin A, in the form of carotene and fish liver concentrate. Since the former, because of its color, will impart a red-brown coloration to the isoamyl alcohol and the latter a dark brown coloration to the same solvent, it is advisable, when a mixture of different vitamins is being tested for thiamine by this procedure, to remove the vitamin A first by shaking the aqueous suspension of these substances with either isobutyl or isoamyl alcohol before proceeding with the determination for thiamine.

2. Vitamin D, as viosterol in oil. This substance did not produce any interfering color.

3. Ascorbic acid when present in concentrations of 2 to 5 mg. per 10 cc. of solution to be tested will either entirely or partially prevent the formation of the color formed by the reaction of thiamine on the reagent. This might be expected because of the reducing properties of vitamin C (3, 10). An interfering color is not produced by the ascorbic acid. For the determination of thiamine in the presence of ascorbic acid, the following procedure is recommended. To an appropriate amount of the solution to be tested add distilled water to bring the volume to about 20 cc. Adjust the

pH to 4.5 with acetic acid. The vitamin C is then oxidized with 1 per cent potassium permanganate, which is added to a faint color. The thiamine will not be oxidized under these conditions in such short a time (11). The slight excess of the potassium permanganate is decolorized with about 2 cc. of the 1 per cent solution of sodium bisulfite. The 5 cc. of the diazo solution are added next and the rest of the procedure follows the general directions for the determination of the thiamine. The results obtained by this procedure are given in Table II, showing that there is no loss in thiamine when this modification is employed.

4. Riboflavin. This factor neither prevents the color formation nor does it produce an interfering color.

TABLE II

Quantitative Recovery of Thiamine after Treatment with $KMnO_4$ and $NaHSO_3$

Sample No.[*]	Thiamine added	Thiamine found	Ascorbic acid added
	units	*units*	*mg.*
1	20	20	0
2	20	4	1
3	20	19	1
4	20	20	1
5	10	10	1
6	5	5	1
7	10	9	0
8	20	19	0

[*] Samples 3 to 8 were treated with 1 per cent $KMnO_4$ and $NaHSO_3$.

5. The other components of the vitamin B complex, such as vitamins B_3, B_4, B_5, pyridoxine, nicotinic acid, as well as pantothenic acid, did not produce or prevent the formation of any color on reaction with the reagent.

Influence of pH on Reaction—Adjustment of the pH of solutions of thiamine to as low as 4 prior to the addition of the diazo solution and the subsequent adjustment of the pH to 7.5 to 8 gave 98 to 100 per cent recovery of the thiamine. Pure aqueous solutions of varying concentrations of thiamine chloride were tested. The results are shown in Table III. If solutions were made too alkaline, losses of about 50 per cent occurred, which may be due to the destruction of the thiamine or the resulting colored compound. It will be recalled that the thiazole fraction of the thiamine molecule also produces a red color with the reagent, but of much less intensity than the vitamin itself.

Time Required for Development of Maximum Color—20 units of thiamine were tested with the reagent and the color produced was extracted with isoamyl alcohol after various periods, ranging from 1 minute to 24 hours,

the latter sample being used for comparison. As shown in Fig. 2, the reaction was completed within 2 minutes.

Effect of Variations in Quantities of Thiamine and Reagent—In one series of tests from 1 to 100 units of thiamine was used, while 5 cc. of the diazo

TABLE III

Effect of pH on Reaction

Sample No.	pH before addition of diazo solution	pH after addition of diazo solution	Thiamine found	Per cent recovered
			units	
1	2.5	7.5	15	75
2	3.5	7.5	15	75
3	4.0	7.5	19	95
4	4.5	7.5	20	100
5	5.0	7.5	20	100
6	5.5	7.5	20	100
7	6.0	7.5	20	100
8	6.5	7.5	19	95
9	7.0	7.5	10	50
10	7.5	9.0	2	10

20 units of thiamine were added to each sample. Each sample was allowed to stand for 2 minutes before the addition of the isoamyl alcohol and then overnight before the separation of the alcoholic layer.
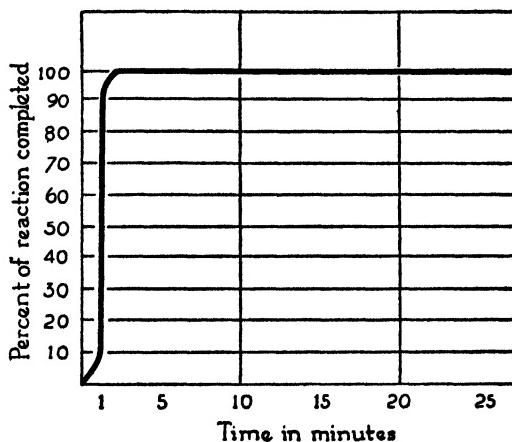
FIG. 2. Time required for maximum color development of thiamine with reagent

solution were used. None of the other factors influencing the reaction was varied. In another series the amount of the thiamine was kept constant, while the amount of the diazo solution was changed. Within certain limits of these variations in the concentrations investigated, no appreciable deviations were observed. Tables IV and V show these results.

Reaction of Reagent with Free Thiamine Only—Vitamin B_1 has been shown to exist in nature as the pyrophosphate to constitute cocarboxylase (12). Tests conducted on fresh solutions of cocarboxylase (Merck) showed that this compound did not react with the diazotized ethyl *p*-aminobenzoate to produce a color, as in the case of free thiamine. This is of great importance, therefore, since solutions containing both the thiamine and the pyrophosphate ester can be assayed for both by difference. In

TABLE IV

Influence of Varying Amounts of Reagent

Sample No.	Thiamine found (20 units added to each sample)	Diazo solution added
	units	*cc.*
1	20	5
2	19	10
3	20	15
4	18	20
5	17	25

In each of the tests the solutions were allowed to stand for 2 to 3 minutes and the color was then extracted with the isoamyl alcohol.

TABLE V

Effect of Varying Amounts of Thiamine

Sample No	Thiamine added	Thiamine found
	units	*units*
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
6	10	10
7	20	20
8	40	39
9	80	78
10	100	96

order to determine the latter, however, it is necessary to hydrolyze it quantitatively to the free thiamine.

Cocarboxylase can be converted into the monophosphate only by acid hydrolysis. This latter compound likewise does not react with the reagent to produce a colored compound. Alkaline hydrolysis is impractical because of the accompanying destruction of the rest of the thiamine molecule.

Enzymatic hydrolysis converts the cocarboxylase to the free thiamine

without any destruction of the thiamine molecule. As one of the numerous sources for phosphatase (12–15) yeast can be used under proper conditions.

Evidence of Enzymatic Conversion of Cocarboxylase to Free Thiamine by Yeast or by Glycerol-Yeast Extract—When solutions of cocarboxylase were heated with fresh live yeast for about 2 to 3 minutes at a temperature of boiling water, quantitative hydrolysis to the free vitamin took place. The vitamin B_1, as found in yeast, is present to a larger extent as the pyrophosphate ester. In the analysis of yeast, it was observed that 30 per cent ethyl alcohol was the best solvent to use. The reaction goes to completion within a very short time. A solution of cocarboxylase in 30 per cent ethyl alcohol containing 1 cc. of a glycerol extract shows that a quantitative hydrolysis of the cocarboxylase to the free thiamine takes

TABLE VI

Effect of Optimal pH on Hydrolysis of Cocarboxylase

pH	Cocarboxylase found (20.12 units added)
	units
8	16
7	20
6	21
5	20
4	21
3.1	20
2	2
1.5	2

3 γ of thiamine = 1 unit of thiamine; 10 γ of cocarboxylase = 7.1 γ of thiamine. The reaction mixtures were allowed to stand for 1 hour, were then filtered, and the filtrate tested for thiamine.

place within an hour. The enzyme was found to be active at pH 3 to 7. These results are shown in Table VI.

A series of tests was carried out to ascertain what percentage of ethyl alcohol was best to use as solvent. To five tubes (Tubes 1 to 5) containing 10 cc. of ethyl alcohol of various concentrations, there were added 20 units of cocarboxylase and 1 cc. of the phosphatase preparation. Another set of five tubes (Tubes 6 to 10) contained the alcohol and the cocarboxylase only. One series was adjusted to pH 3 and another to pH 7. From the results shown in Table VII, it is evident that the 30 per cent alcohol is the best solvent to use.

The temperature and the time influencing the hydrolysis are interesting. If yeast was suspended in distilled water and this suspension was heated for about 3 minutes in boiling water, the same results were obtained as with

the fermentation method of Schultz, Atkin, and Frey (8). Identical results were found when 30 per cent ethyl alcohol was substituted for the distilled water with one noticeable exception in that the samples treated with the alcohol did give the same results whether the suspensions were heated or not. When distilled water is used for the yeast suspensions, temperatures around 90° are needed, however, as shown by the results cited in Tables VIII to X.

Preparation of Phosphatase Solution—1000 gm. of fresh bakers' yeast (Fleischmann's) are stirred with 500 cc. of distilled water and allowed to stand at room temperature for about 1 hour. Enough dry ice is then added

TABLE VII

Effect of Optimal Concentration of Ethyl Alcohol on Hydrolysis of Cocarboxylase by Yeast Phosphatase

Tube No.	Series A, pH 3		Series B, pH 7	
	Alcohol	Cocarboxylase found (20 units added)	Alcohol	Cocarboxylase found (20 units added)
	per cent	*units*	*per cent*	*units*
1	75	2.5	75	2.5
2	50	2.5	50	2.5
3	30	20	30	20
4	20	16	20	15
5	0	2	0	2.5
6	75	2	75	2
7	50	2.5	50	2.5
8	30	2	30	2
9	20	2.5	20	2.5
10	0	2	0	2 5

The mixtures were allowed to stand 1 hour before the estimation of the free thiamine formed was carried out. 3 γ of thiamine = 1 unit of thiamine; 10 γ of cocarboxylase = 7.1 γ of thiamine; 4.22 γ of cocarboxylase = 1 unit of thiamine.

to freeze the mixture, which is then allowed to warm up gradually to room temperature. 1 liter of glycerol is then added and the mixture is stirred for a few minutes and transferred to a cylindrical container. After being kept in the refrigerator overnight, the suspension is centrifuged and the supernatant liquid used as the phosphatase preparation for the hydrolysis of the cocarboxylase.

Modification of Method As Applied to Estimation of Thiamine in Urine—With a slight modification this procedure has been applied to the estimation of thiamine in urine. The test is carried out on urine directly without previous adsorption of the thiamine on permutit or zeolite. Results obtained by this method are comparable with those obtained by various

other investigators using the different methods that are employed at present. Quantities as low as 3 γ of thiamine per 5 cc. of urine can be determined.

TABLE VIII

Influence of Temperature on Hydrolysis of Yeast Cocarboxylase

2 gm. of yeast were suspended in 20 cc. of distilled water, and the suspensions were heated at different temperatures for various periods.

Sample No.	Temperature of water bath	Heating time	Thiamine per cake (12.5 gm.)
	°C.	min.	units
1	98	30	150
2	98	5	187.5
3	98	3	225
4	98	3	200
5	98	2	225
6	98	2	212
7	98	2	200
8	98	2	218.5*
9	98	2	218.5†
10	95	3	191.75
11	90	3	225
12	90	3	212
13	90	2	191.5
14	90	1.5	200
15	85	3	218
16	85	2	212.5
17	85	1	191.5
18	82–83	2	168.5
19	80	3	162.5
20	80	2	150
21	80	1	112
22	70	3	37.5
23	70	2	37.5
24	50	45	
25	45	60	

The suspensions were filtered immediately after the heating and 10 cc. of the filtrate were used for each analysis.

* The suspension was allowed to stand an additional 30 minutes after the heating before it was filtered.

† The suspension was cooled to 10° with ice water immediately after the heating and then filtered.

The urine is collected under toluene and the pH is adjusted to about 3 with sulfuric acid. The sample is then concentrated *in vacuo* at a temperature of about 50–60° to about one-tenth of its original volume. Any precipitate in the residue is filtered off and aliquot portions of the filtrate are

shaken with isobutyl alcohol. 5 or 10 cc. portions of the concentrated urine, so treated, are then used for the estimation of thiamine as follows: (a) Dilute to 20 cc. with distilled water; (b) adjust the pH to 5.5 to 6; (c)

TABLE IX

Analysis of Free Thiamine in Yeast

	Thiamine per cake (12.5 gm.)
	units
Ethyl acetate 0.5 cc.........	75
" " 0.25 "	62.5
Capryl alcohol 0.1 "	43.75
Sand 2 gm.................................	72.5
" 2 "	50
" 2 "	75
" 2 "	50
Yeast frozen in refrigerator 72 hrs................	56.5
" " " " 48 "	56.5

2 gm. of yeast were suspended in 20 cc. of water; 10 cc. of the filtrate representing 1 gm. of yeast were used for the test. Prior to the addition of the water, the above substances were added to the yeast, or it was frozen in the refrigerator. These samples showed only about 25 per cent of the amount of the thiamine found as those treated as reported in Table VIII. These results confirm those of Melnick and Field (4).

TABLE X

Analysis of Free Thiamine and Cocarboxylase in Yeast

Sample No.	Yeast A		Yeast B		Yeast C	
	units per gm.	*units per cake*	*units per gm.*	*units per cake*	*units per gm.*	*units per cake*
1	14	175	15	187.5	17	212.5
2	14	175	15	187.5	17	212.5
3	15	187.5	15	187.5	17	212.5
4	21		21		23	
5	4	50	4	50	5	62.5

2 gm. of yeast were suspended in 20 cc. of liquid; the test was carried out on 10 cc. of the filtrate representing 1 gm. of yeast. The samples were treated as follows: Sample 1, boiled in 30 per cent alcohol for 2 minutes; Sample 2, boiled in water for 3 minutes; Sample 3, yeast rubbed with sand and suspended in 30 per cent alcohol; Sample 4, yeast rubbed with sand and suspended in 30 per cent alcohol containing 6 units of cocarboxylase per 10 cc. of alcohol (4.22 γ of cocarboxylase = 3 γ of thiamine = 1 unit of thiamine); Sample 5, yeast rubbed with sand and suspended in water containing 6 units of cocarboxylase per 10 cc. of water.

add 1 per cent $KMnO_4$ to the appearance of the pink color to oxidize any ascorbic acid present; (d) add 2 cc. of freshly prepared 1 per cent sodium

bisulfite solution; (e) follow with 5 cc. of the diazo solution and complete the reaction in the usual manner.

Before the separation of the isoamyl alcohol, the reaction mixture is allowed to stand for about 8 hours so that the interfering color, which may be formed, will disappear. Should there still be some of these colors present at the end of that time, the alcohol after separation is then shaken with a small amount of distilled water. This last procedure is not necessary very often. Concentration of urine from persons on a high thiamine diet is not necessary.

Results shown in Table XI indicate a 98 per cent recovery of thiamine added to urine.

TABLE XI

Recovery of Thiamine Added to Urine

Subject No.	Volume of urine for 24 hrs.	Concentrated to	Thiamine in 5 cc., concentrated	Thiamine in 5 cc., concentrated; 10 units added	Thiamine in 24 hr. urine
	cc.	*cc.*	*units*	*units*	*units*
I	1450	150	2	12	60
			2	11	60
II	850	125	3	12	75
			3	13	75
III	640	120	6	14	144
			6	16	144
IV	620	100	4	14	80
			4	14	80
V	470	125	2	11	50
			2	12	50

DISCUSSION

The reagent as employed seems to be specific for the 4-methyl-5-β-hydroxyethylthiazole part of the thiamine molecule. A quantitative procedure has been developed whereby the reagent can be used for the estimation of thiamine without the preliminary isolation of the thiamine by the use of adsorbates. When the latter are used, it is rather difficult to obtain 100 per cent recovery of the vitamin.

The reagent does not react with cocarboxylase, the pyrophosphate of thiamine, to give a colored compound. The ester has been hydrolyzed quantitatively by yeast phosphatase in 30 per cent ethyl alcohol, both at room temperature and at temperatures up to 98°. With water in place of the alcohol, temperatures above 85° seem to be necessary. Melnick and Field ((4) p. 535) state: "At 70° no hydrolysis is possible owing to the

inactivation of the enzyme by heat." They kept their yeast suspension at 70° for 1 hour and that period may be sufficient to destroy all the phosphatase. On the other hand, some of the cocarboxylase should have been converted to the free vitamin. It is not clear why they were unable to detect any free thiamine after their treatment at that temperature.

In our experiments, temperatures much higher than 70° were reached. The suspensions were kept at those higher temperatures for a relatively short time, however. Quantitative conversion of the ester to the free vitamin took place almost instantaneously under the experimental conditions employed by us. A possible interpretation of this phenomenon may be offered. (1) Heat breaks the yeast cells, liberating the phosphatase and the cocarboxylase, the latter being converted to the free thiamine. (2) The rate of hydrolysis proceeds at a much greater speed than the rate of destruction of the enzyme, which, no doubt, takes place because of the destruction of enzymes by heat in general. (3) The free thiamine formed is stable enough at that temperature for such a short period. It requires longer heating to destroy the vitamin B_1.

SUMMARY

A chemical method for the determination of thiamine has been described by which diazotized ethyl p-aminobenzoate reacts with the vitamin to form a colored compound which can be extracted quantitatively from aqueous solution by the use of isoamyl alcohol.

The specificity of the reagent, the influence of the pH on the reaction, the time necessary to complete the reaction, and the reproducibility of the method have been described.

A modification of the procedure in the presence of ascorbic acid has been described.

The method is specific for free thiamine. The pyrophosphate of thiamine will not give the test. The ester must first be hydrolyzed to the free vitamin. In the case of yeast preparations, this can be accomplished very simply by the action of the phosphatase in the yeast. This enzyme is liberated from the yeast cells by heating a yeast suspension in water or 30 per cent ethanol at a temperature of boiling water for 2 to 3 minutes.

The preparation of a glycerol phosphatase solution has been described.

Under the conditions described, the cocarboxylase can be converted quantitatively to the free thiamine at a pH range from 3 to 7 during a relatively short period.

A modification of the procedure has been described to make the test applicable to the estimation of thiamine in urine, without any adsorption of the vitamin.

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THE ACONITE ALKALOIDS

VIII. ON ATISINE

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The roots of the Indian plant *Aconitum heterophyllum*, or atis, have long been known to contain an alkaloid, atisine (1), which belongs in the category of simpler aconite alkaloids or alkamines of low toxicity. The possibility of a structural relationship between this group of alkaloids and the more complicated highly toxic aconitine group makes a study of their chemistry of particular importance. In recent work Lawson and Topps (2) have reported the results of their study of this alkaloid. They revised the older formulation of Wright (3) and of Jowett (4) of $C_{22}H_{31}O_2N$ to $C_{22}H_{33}O_2N$ and showed that atisine contains an N-methyl group but no methoxyl group. However, their conclusion that a methylenedioxy group is present we have found to be incorrect. Our present work confirms their observations in some respects but in others we have been forced to different conclusions.

Atis root of commercial origin was obtained from India.[1] The isolation of atisine as the hydrochloride followed with minor modifications the procedure used by Lawson and Topps. It was found necessary to employ an excess of NaOH to liberate the alkaloid before extraction of the final crude aqueous mixture. 12 kilos of the root were found to yield about 98 gm. of crude hydrochloride. Analysis of the recrystallized salt supported the formulation $C_{22}H_{33}O_2N \cdot HCl$. The alkaloid itself could not be obtained in crystalline form. It distilled readily in a molecular still. Analysis of such material supported the formulation $C_{22}H_{33}O_2N$. The hydrochloride as obtained by us showed the rotation $[\alpha]_D^{25} = +28°$ ($c = 1.1$ in H_2O). This differed considerably from the figures published originally by Jowett; *viz.*, $[\alpha]_D = +18.46°$. A possible explanation for this discrepancy has since been found in the instability of atisine in alkaline solution. Even when the alkaloid as the free base was allowed to stand in alcoholic solution at room temperature, a slow change was found to occur. When, after a number of days, the base was reconverted into the hydrochloride, the latter now showed $[\alpha]_D^{25} = +13.5°$. This change was accelerated by the addition of alkali and led to the formation in part of the substance to be described

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[1] The rhizomes of commercial *Aconitum heterophyllum* were purchased from an Indian source through the kind aid of Dr. R. T. Major of Merck and Company, Inc., Rahway, New Jersey.

below.   In the procedure used by us for the isolation of the alkaloid from the plant extract the free base was promptly extracted with benzene after liberation with alkali.   It is probable that the material for which Jowett reported his rotations was the product of partial rearrangement.   This rearrangement may be due to the double bonds of atisine, since the tetrahydro derivative to be described below was not similarly affected by alkali.

Lawson and Topps have described the production of a so called demethylated alkaloid, $C_{21}H_{31}O_2N$ (m.p. 147°) by the action of potassium ethoxide on atisine.   On repeating the brief directions of these workers we have obtained undoubtedly the substance described by them, but it was found to have retained the N-methyl group of atisine.   The same substance was produced by heating the alkaloid in methyl alcoholic NaOH.   Although an apparent mixture of substances resulted, the crystalline base was readily isolated first as the hydrochloride.   Analyses of the base and its hydrochloride corresponded with the figures for a dihydroatisine, $C_{22}H_{35}O_2N$. On hydrogenation either the base or the hydrochloride was found to absorb but 1 mole of H_2 with the formation of the tetrahydroatisine described below.   The production of such a substance from atisine if a dihydro derivative would appear to be the result of a disproportionation of hydrogen. The possibility that atisine itself is really a mixture and that such a dihydro derivative had its origin in one of the components of such a mixture, while still to be considered, appears to be much less likely.

The base atisine, as well as its tetrahydro derivative, was found to possess 2 active H atoms.   These must be contained in two hydroxyl groups, since *diacetylatisine hydrochloride* was formed on acylation of atisine hydrochloride.   Thus the oxygen atoms of atisine have been accounted for by OH groups and not by a methylenedioxy group as suggested by Lawson and Topps.[2]

Atisine on hydrogenation with platinum oxide catalyst absorbed 2 moles of H_2 presumably due to two double bonds.   The resulting product proved to be a mixture of apparently isomeric *tetrahydroatisines* which, unlike atisine itself, readily crystallized.   One of these isomers was easily separated because of its sparing solubility and melted at 171–174°.   In toluene $[\alpha]_D^{25} = -33°$; in chloroform $[\alpha]_D^{25} = -23°$.   The same substance was obtained from the dihydroatisine recorded above.   Unlike atisine, the tetrahydro derivative is stable toward alkali.

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[2] These workers reported that the Zerewitinoff determination with atisine hydrochloride was negative.   In our repetition of the determination with the salt we have also found no appreciable evolution of CH_4 at 25°.   However, at 95° 0.472 per cent of active H was found.   The unsatisfactory behavior of this salt is undoubtedly due to physical complications.

Lawson and Topps reported the hydrogenation of atisine hydrochloride in acetic acid with palladium black to yield a dihydroatisine which was described only as the hydrochloride and concluded that atisine contains but one double bond. In repeating this experiment we found that the hydrogenation with palladium proceeded more slowly than with platinum oxide catalyst but nevertheless continued into the 2 mole stage. From the resulting mixture the above tetrahydroatisine was readily obtained. In an experiment in which the hydrogenation with palladium was interrupted at the 1 mole stage a mixture was obtained which contained unreacted atisine and from which tetrahydroatisine was isolated.

When the attempt was made to reduce atisine with sodium and alcohol, the only crystalline substance which could be isolated appeared to be identical with dihydroatisine. When this substance was in turn catalytically hydrogenated, it yielded a mixture from which the above tetrahydroatisine was isolated. Whether the production of dihydroatisine in this case was due to the reducing action of the sodium and alcohol or due to the transformation of atisine by the resulting sodium ethylate was not determined.

On the assumption of the correctness of the formulation $C_{22}H_{33}O_2N$ for atisine, the presence of two hydroxyl groups and two double bonds would indicate that its skeletal structure must be of pentacyclic nature. Since it contains an N-methyl group and apparently at least one C-methyl group, as shown by the Kuhn and Roth (5) determination, such a saturated system cannot be greater than $C_{20}H_{33}N$. It is probable that three of these rings consist of a hydrogenated phenanthrene. This was supported by the results of dehydrogenation studies.

In the repetition of the dehydrogenation of atisine with selenium already reported by Lawson and Topps we have confirmed in an important respect the observations of these workers. The dehydrogenation product which appeared to be formed in largest amount was the hydrocarbon $C_{17}H_{16}$ reported by them. Their conclusion that it is a substituted phenanthrene hydrocarbon has been supported by absorption spectra measurements carried out at The Rockefeller Institute for Medical Research by Dr. George I. Lavin as shown in Fig. 1. We have previously described (6) the isolation of phenanthrene hydrocarbons from the new aconite alkaloid staphisine and the study of their absorption spectra. The curves of the latter[3] are given, also in Fig. 1, for comparison with the curve of the $C_{17}H_{16}$ hydrocarbon from atisine.

[3] It has since been found that the values of the extinction coefficients as plotted in the curves given in the original paper on staphisine (6) were somewhat too high because of a miscalculation of data. The corrected values are plotted in the curves of Fig. 1.

From the hydrocarbon fraction a substance $C_{15}H_{12}$ has been obtained which agreed in properties with synthetic 1-methylphenanthrene. This was confirmed by comparison of their picrates. From a higher fraction an oily *hydrocarbon* $C_{18}H_{18}$ was isolated and characterized as the *trinitrobenzene* derivative (163–166°) and the *picrate* which melted at 153–156°.
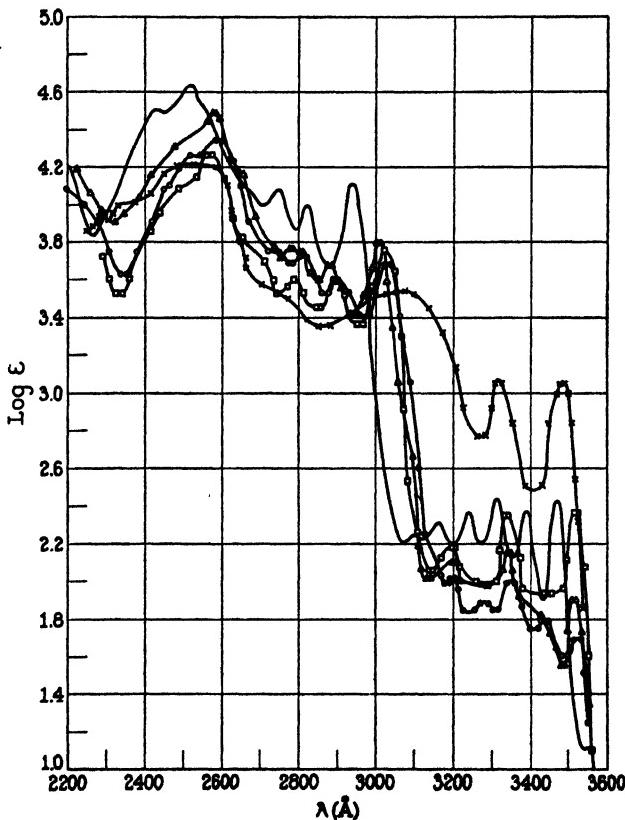
Fig. 1. Absorption spectrum curves in ethyl alcohol solution. The solid curve = phenanthrene; $\triangle$ = $C_{16}H_{14}$ hydrocarbon from staphisine; $\bigcirc$ = $C_{19}H_{20}$ hydrocarbon from staphisine; $\square$ = $C_{17}H_{16}$ hydrocarbon from atisine; $\times$ = $C_{16}H_{15}N$ base from atisine. ϵ is the molecular extinction coefficient.

The base $C_{17}H_{17}N$ described by Lawson and Topps as a product from the so called demethylated atisine (dihydroatisine) has not been encountered by us among the products from atisine. Instead a *base* $C_{18}H_{15}N$ which melted at 83–85° was obtained. This in turn gave a *picrate* (221–223°) and a *methiodide* which melted at 233–235°. The absorption spectrum of the base is recorded in Fig. 1.

A possible oxygen derivative of the latter base, the *base* $C_{18}H_{15}ON$, was

also obtained which melted at 258–261°.   In addition, as recorded in the experimental part, two other crystalline substances and a basic oil isolated as a picrate were encountered in amounts too small for proper and conclusive characterization.

EXPERIMENTAL

Atisine—The isolation of the alkaloid from atis root has been based essentially on that of Jowett and of Lawson and Topps, with a few modifications.

13 kilos of ground root were extracted twice with 39 liters of 70 per cent alcohol.   In each case the extracted root was squeezed dry in a press. The extract was concentrated under diminished pressure to remove the alcohol and the remaining thin aqueous syrup was treated with one-fourth of its volume of 10 per cent H_2SO_4.   The mixture, which was strongly acid to Congo red, was extracted with chloroform several times and the remaining clear aqueous solution was carefully treated with Na_2CO_3 solution until slightly alkaline to litmus.   The mixture was extracted repeatedly with benzene and the extract was set aside.   The aqueous phase was then shaken with fresh benzene, made strongly alkaline with NaOH solution, and promptly and repeatedly extracted with benzene.   The washed and dried benzene extract on concentration yielde d a light brown resin of mixed alkaloids.   This was dissolved in alcohol and, while chilling, carefully treated with HCl (1.19) until barely acid to Congo red.   During this operation a copious mass of colorless needles formed which were collected with 95 per cent alcohol.   35.3 gm. were obtained.   On concentration of the mother liquor successive additional crops of crystals brought the yield to 46 gm.   In a later experiment 24 kilos yielded four crops of crystals which totaled 98 gm.   Analyses of these fractions indicated that they consisted essentially of atisine hydrochloride.   From the ultimate mother liquors on longer standing additional material was obtained which was found to consist of salts of at least two new alkaloids as described in Paper IX.

After recrystallization from 50 per cent alcohol the salt formed flat needles which melted with decomposition at 311–312° (uncorrected).

$$[\alpha]_D^{25} = +28° \ (c = 1.10 \text{ in } H_2O)$$

$C_{22}H_{34}O_2NCl$.   Calculated.   C 69.52, H 9.02
Found.   (*a*) " 69.92, " 9.16
"   (*b*) " 69.74, " 8.79
"   (*c*) " 69.50, " 9.06
"   (*d*) " 69.78, " 8.79

Attempts to crystallize the free base obtained by decomposition of the salt were not successful.   However, the residue obtained on concentration of the benzene solution of the base distilled rapidly and quantitatively

under 0.01 μ and at a bath temperature of 140°. It formed a white opaque film on the condenser which could be removed as a powder. It melted at 57–60°.

$C_{22}H_{33}O_2N$. Calculated, C 76.91, H 9.69; found, C 76.72, H 9.65

The molecular weight was determined in camphor by the method of Rast. Calculated, 343.27; found, 342.5.

A C-methyl determination according to Kuhn and Roth (5) was as follows: 0.0376 gm. of base after three distillations consumed a total of 0.74 cc. of 0.105 N NaOH. Calculated for one CH_3, 1.045 cc.

The Tschugaeff-Zerewitinoff determination was as follows: 11.92 mg. of base gave 1.45 cc. of CH_4 (26°, 739 mm.). At 95° there was no change. Found, H 0.487; calculated for 2H, 0.587.

Diacetylatisine Hydrochloride—0.1 gm. of atisine hydrochloride and 2 cc. of acetic anhydride were refluxed for 10 minutes. The alkaloid salt gradually dissolved. The clear solution was repeatedly concentrated *in vacuo* after addition of alcohol. The reaction product crystallized from alcohol-ether in delicate needles which melted at 241–243° (uncorrected) with decomposition.

$C_{26}H_{38}O_4NCl$. Calculated. C 67.27, H 8.26
                        Found. (a) " 66.97, " 8.54; (b) C 66.89, H 8.53

Atisine and Alkali. Dihydroatisine(?)—3m. of atisine hydrochloride were sealed in a tube with a saturated solution of NaOH (15 to 20 gm.) in 35 cc. of methyl alcohol. The mixture was heated at 100° for 43 hours. The colorless mixture after dilution was extracted with chloroform. The extract was washed and dried over K_2CO_3 and then concentrated. Residual chloroform was boiled off with 95 per cent alcohol. On careful addition of HCl (1.19) until the mixture was just acid to Congo red, the hydrochloride rapidly crystallized. After collection with cold alcohol 1.36 gm. of the salt were obtained. A portion of this salt on recrystallization by solution in hot 95 per cent alcohol followed by concentration separated as thin or broad flat needles which melted with decomposition at 261–263°.

$$[\alpha]_D^{27} = -16° \ (c = 1.02 \text{ in } H_2O)$$
$C_{22}H_{35}O_2N \cdot HCl$. Calculated. C 69.16, H 9.50
                            Found. (a) " 69.23, " 9.29; C 69.01, H 9.36
                            " (b) " 69.32, " 9.50

The major portion of the above unrecrystallized salt was directly decomposed with dilute NaOH and the base was extracted with ether. The dried extract on concentration readily yielded large, well formed prisms of

the base which melted at 150–153° after preliminary sintering. The micro melting point was 156–158° (corrected).

$$[\alpha]_D^{28} = -45° \ (c = 1.02 \text{ in toluene})$$

$C_{22}H_{35}O_2N$. Calculated. C 76.46, H 10.22, N(CH_3) 4.35
Found. (a) " 76.42, " 10.11; C 76.35, H 10.07
" (b) " 76.74, " 10.27, N(CH_3) 2.64

A portion of the base was reconverted into the hydrochloride. The latter again crystallized from alcohol-ether in needles which melted at 261–262°.

$$[\alpha]_D^{20} = -17° \ (c = 1.00 \text{ in } H_2O)$$
Found. C 69.20, H 9.62

The attempt was made to repeat the reported demethylation of atisine as described by Lawson and Topps. Potassium ethylate was prepared from 1.4 gm. of K and 19 cc. of absolute alcohol in an atmosphere of nitrogen. 0.5 gm. of atisine hydrochloride was then added and the mixture was refluxed for 5.5 hours. During the operation much of the alcohol was carried off by the nitrogen, so that the actual mixture became more concentrated, which more than compensated for the partial neutralization of the HCl. After dilution the dried ether extract yielded a colorless resin which did not crystallize directly. It was converted into the HCl salt, several fractions of which crystallized readily. The salt formed needles from alcohol and melted with effervescence at 303° (uncorrected).

$$[\alpha]_D^{27} = -2° \ (c = 0.98 \text{ in } H_2O)$$
Found. C 69.61, H 8.95, N(CH_3) 2.37

A second fraction was obtained from the mother liquor. Found, N(CH_3) 2.55.

The above melting point, rotation, and analytical data definitely point to a mixture. When the base was recovered from the salt with alkali, the ether extract on concentration readily crystallized as characteristic prisms which melted at 152° and were identical with the above base obtained with alcoholic NaOH.

$C_{22}H_{35}O_2N$. Calculated. C 76.46, H 10.22; Found, C 76.51, H 10.01

Tetrahydroatisine—0.25 gm. of atisine hydrochloride was hydrogenated with 50 mg. of platinum oxide catalyst in methyl alcoholic solution under approximately 3 atmospheres pressure. The reaction was rapid and practically completed within 10 minutes after a total of 45 cc. of H_2 or about 32 cc. by the substance was absorbed. Calculated for 2 moles of H_2, 31.5 cc. After filtration and removal of the solvent the free base was liberated with alkali and extracted with ether. The extract on concentration to small volume readily crystallized after chilling. 0.145 gm. of substance

was collected in the cold with ether. It melted partly at 155–156°, but did not clear until above 165°.

$C_{22}H_{37}O_2N$. Calculated, C 76.02, H 10.74; found, C 76.21, H 10.85

This was shown to be a mixture. On recrystallization from 95 per cent alcohol it crystallized as flat needles which melted at 171–174°. The melting point was not changed by further recrystallization. The yield was about one-third of the starting material.

Found. (a) C 75.90, H 10.52; (b) C 76.37, H 10.68
$[\alpha]_D^{25}$ = $-33°$ (c = 1.03 in toluene); $-23°$ (c = 1.00 in chloroform)

The Tschugaeff-Zerewitinoff determination was as follows: 11.53 mg. gave 1.68 cc. of CH_4 (25°, 750 mm.); at 95° there was no change. Found, H 0.593; calculated, 2H 0.581.

From the original ether mother liquor on further concentration additional crystallization was obtained in small amount (11 mg.) which consisted mostly of small glistening prisms. The melting point also indicated non-homogeneity. It began to soften above 155° but did not form a clear melt under 168°.

$[\alpha]_D^{25}$ = $-57°$ (c = 0.77 in toluene)
Found. C 76.32, H 10.68

0.25 gm. of atisine hydrochloride was hydrogenated in acetic acid solution with 50 mg. of Pd black under 3 atmospheres pressure. The absorption was much slower than in the above case with platinum catalyst. Although after 6 hours about 1 mole in excess of that required by the catalyst had been absorbed, absorption continued and after 24 hours was interrupted. 28 cc. of H_2 were absorbed by the substance.

After liberation of the base it crystallized from ether as an obvious mixture of isomers which gradually and incompletely melted at 138–139° and became clear somewhat higher. Found, C 76.10, H 10.57.

After repeated recrystallization from 95 per cent alcohol, it melted at 171–174° and showed no depression when mixed with the above tetrahydroatisine obtained with platinum catalyst.

In an experiment in which the hydrogenation with Pd was interrupted at the 1 mole stage the only product which crystallized as the free base was obtained in small yield. Found, C 76.16, H 10.65.

After repeated recrystallization from alcohol, it melted at 171–174° and showed no depression with tetrahydroatisine.

0.1 gm. of dihydroatisine with 50 mg. of platinum oxide catalyst in methanol rapidly absorbed 1 mole of H_2 in excess of the requirements of the catalyst. The crystalline reaction product proved to be a mixture which after several recrystallizations from alcohol gave 23 mg. of characteristic

needles of the tetrahydro derivative which melted at 172–173°. Found,
·C 76.02, H 10.50.

A similar result was obtained when the hydrochloride of dihydroatisine
was hydrogenated. The hydrogenated base recovered from the reaction
mixture after repeated recrystallization melted at 172–173°. Found, C
76.29, H 10.73.

Atisine and Sodium—2 gm. of sodium were added in portions to a re-
fluxing solution of 0.5 gm. of atisine hydrochloride in 30 cc. of absolute
alcohol. The diluted reaction mixture was extracted with ether. The
washed, dried, and concentrated extract on careful addition of petroleum
ether gradually crystallized. 0.14 gm. of glistening, flat, often diamond-
shaped prisms was obtained which after recrystallization melted at 153–
155°. A mixture with dihydroatisine melted at 150–153°.

$$[\alpha]_D^{\text{P}} = -45.5° \ (c = 1.01 \text{ in toluene})$$

$C_{22}H_{35}O_2N.$ Calculated.   C 76.46, H 10.22, N(CH_3) 4.35
          Found. (*a*)   " 76.26, " 10.08
             " (*b*)   " 76.11, " 10.06, N(CH_3) 2.74
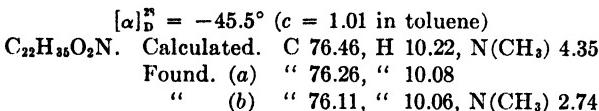
The Tschugaeff-Zerewitinoff determination was as follows: 15.475 mg.
gave 2.0 cc. of CH_4 (26°, 739 mm.); there was no change at 95°. Found,
H 0.517; calculated for 2H, 0.584.

0.1 gm. of this substance was hydrogenated with 50 mg. of platinum
oxide catalyst in methyl alcohol. The substance absorbed 10 cc. of H_2.
The resulting base crystallized as needles from alcohol, melted at 170–172°,
and showed no depression when mixed with tetrahydroatisine.

$C_{22}H_{37}O_2N.$ Calculated, C 76.02, H 10.74; found, C 76.39, H 10.67

12.90 mg. of substance gave 1.78 cc. of CH_4 (26°, 739 mm.); there was
no change at 95°. Found, H 0.552; calculated, 2H 0.581.

Dehydrogenation of Atisine—18 gm. of atisine hydrochloride were de-
composed with NaOH solution and the free base was extracted with ether.
The dried extract was concentrated to dryness in a dehydrogenation ap-
paratus. 50 gm. of selenium powder were then added, and the mixture
was heated in a current of nitrogen for 2 hours in a salt bath kept at 340°.

The resulting distillate was examined for basic, phenolic basic, phenolic,
and neutral fractions. Only a small amount of basic material was ob-
tained, which had the odor of pyridine derivatives, but nothing crystalline
could be isolated. Appreciable amounts of phenolic or basic phenolic frac-
tions were not present. The neutral fraction contained more material but
appeared to consist largely of unstable selenium derivatives of unpromising
nature.

The residue which remained in the dehydrogenation flask was finely
ground and exhaustively extracted with ether. Evaporation of the extract

yielded a residue of about 11 gm. It was redissolved in ether and 35 cc.
of 10 per cent HCl were added. After thorough shaking, the ether phase·
was separated from the acid layer, which contained considerable insoluble
sticky tar. Investigation of the ether solution for acid or phenolic products
proved negative. The dried ether solution yielded a residue of 5.9 gm.
This was dissolved in benzene and passed through a chromatograph pre-
pared with 1 pound of Brockmann's alumina suspended in benzene. Ap-
proximately 3 gm. of material passed through the column with the benzene.
This hydrocarbon fraction was set aside to be treated as described below.
2.6 gm. of material were subsequently eluted with 5 per cent methyl alcohol
in benzene. This fraction was sublimed in an apparatus under 0.2 mm.
pressure up to an oil bath temperature of 250°. 2 gm. of resinous sublimate
were obtained which contained free selenium. This was removed with

TABLE I

Fractionation of Neutral or Weakly Basic Heterocyclic Fraction

Fraction No.	Bath temperature	Column temperature	Weight of fraction	Analysis		
				C	H	N
	°C.	°C.	mg.	per cent	per cent	per cent
1	230	180	50	84.52	10.54	
2	230	180	150		.	
3	245	190	150			
4	255	200	150	80.54	9.20	
5	255	210	150	79.32	9.20	
6	255	218	150	79.98	9.48	
7	266	224	150	80.13	10.00	4.32
8	266	230	150	79.67	9.32	
9	266	238	150	80.21	9.48	

bone-black in ether solution. After removal of the solvent, the residue
was resublimed with heating not higher than 200°. The sublimate weighed
1.8 gm. This was fractionally distilled in a fractionating apparatus with a
column 21 cm. in length as shown in Table I.

Fractions 7 and 8 crystallized from ether. Fraction 8 gave 26 mg. of
delicate curved needles which showed a rather indefinite melting point of
180–190°. Its properties made further purification very difficult, especially
with the small amount of material available.

$C_{21}H_{31}ON$.   Calculated.   C 80.45, H 9.97, N 4.47
$C_{20}H_{29}ON$.   "   " 80.20, " 9.77, " 4.68
                Found.   " 79.90, " 9.80, " 4.21
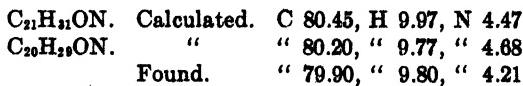
The analytical data approximated the figures for a formulation
$C_{21}H_{31}ON$.

The C figure for the formula $C_{21}H_{31}ON$ is somewhat low, but the fractions from Nos. 4 to 9 more or less approach the theory and it appears possible that this material could have been formed from atisine by the loss of an oxygen atom and a CH_2 group with a rearrangement which rendered the nitrogen atom no longer strongly basic.

The insoluble sticky tar suspended in the above acid extract was extracted with chloroform. The latter was then shaken with 10 per cent NaOH. Solid material separated at the interface and was removed mechanically after standing for some time. The chloroform layer was separately investigated as given below. The solid material was dissolved in sufficient chloroform and the solution was dried over K_2CO_3. Upon evaporation a residue of 220 mg. remained, which was partly crystalline. After successive recrystallizations from acetone and chloroform pale yellow needles were obtained which melted at 258–261°. The analytical data proved to be in agreement with the calculated data for an oxygen derivative of the $C_{16}H_{15}N$ base reported below.

$C_{16}H_{15}ON$.   Calculated. C 80.97, H 6.38, N 5.90
        Found.   " 80.97, " 6.13, " 6.02

The above chloroform layer after drying and concentration yielded a residue which weighed 4.9 gm. It was dissolved in 50 cc. of benzene and chromatographed through 300 gm. of alumina in benzene. Only a small amount of material passed through the column with benzene or with anhydrous ether alone. However, when 5 per cent methyl alcohol was added to the ether, a total of approximately 2.4 gm. of material was eluted from the column. This material was sublimed and all was collected that would distil under 0.2 mm. pressure and an oil bath temperature of 200°. The sublimate weighed 2.2 gm. It was fractionated with a fractionating column 21 cm. in length at 0.2 mm. The record of the fractionation is given in Table II.

Each fraction contained approximately 150 mg. of material. Fraction 5 was sharply crystalline, while Fractions 3 and 4 were largely crystalline. The fractions from Nos. 7 to 11, as directly obtained, were resins, but Fractions 8, 9, and 10 crystallized partially on standing.

The Base $C_{16}H_{15}N$—After two recrystallizations from ether, Fraction 5 formed broad leaves and melted at 83–85°. It did not contain active hydrogen and was therefore a tertiary base.

$C_{16}H_{15}N$.   Calculated. C 86.83, H 6.84, N 6.33
        Found.   " 87.01, " 7.08, " 6.15

The crystalline base with the calculated amount of picric acid gave a picrate from acetone as long well formed needles. It appeared to melt partially and change form under the microscope at 180° with the major

portion of the material melting at approximately 221–223°. A few crystals persisted to a higher temperature.

$C_{16}H_{15}N \cdot C_6H_3O_7N_3$. Calculated, C 58.64, H 4.03; found, C 58.60, H 4.08

20 mg. of the crystalline base were dissolved in 2 cc. of methyl iodide. Crystalline material soon separated. After 4 hours at room temperature the theoretical weight of the methiodide as long rods was collected. The melting point, 233–235°, was not changed by recrystallization.

$C_{17}H_{18}NI$. Calculated, C 56.19, H 5.00; found, C 56.16, H 5.02

Fractions 1 and 2 appeared from the analysis to contain material which was not completely dehydrogenated. Fraction 2 was treated with 170 mg. of picric acid, in 8 cc. of acetone. 70 mg. of yellow needles were ob-

TABLE II

Fractionation of Basic Material

Fraction No.	Bath temperature	Column temperature	n_D^{25}	Analysis	
				C	H
	°C.	°C.		per cent	per cent
1	205	155	1.5596	84.56	9.76
2	210	155	1.5673	84.63	9.77
3	213	155	1.5882	85.03	8.87
4	217	160	1.6300	86.08	7.48
5	217	165	1.6411	86.20	7.61
6	220	165	1.6150	84.15	7.76
7	225	170		81.53	8.95
8	230	180			
9	240	180		80 81	9.63
10	250	200			
11	250	220		80.26	9.00

tained which appeared to consist mostly of the picrate of the $C_{16}H_{15}N$ base. On concentration the mother liquor gave an additional 164 mg. of yellow needles which after recrystallization melted at 210–213°. Analytical data obtained with this material, however, suggested the picrate of a base $C_{20}H_{29}N$.

$C_{20}H_{29}N \cdot C_6H_3O_7N_3$. Calculated, C 60.90, H 6.30; found, C 61.03, H 6.66

Fraction 11 crystallized directly from a concentrated solution of ether. After recrystallization rosettes of needles were obtained which did not possess a sharp melting point. However, the analytical data did not differ much from those directly obtained with Fraction 9 and suggested a formulation of $C_{20}H_{27}ON$.

$C_{20}H_{27}ON$.   Calculated, C 80.75, H 9.15; found, C 80.64, H 9.38

The previously mentioned main hydrocarbon fraction was sublimed at an oil bath temperature of 200° and under 0.2 mm. pressure.   The sublimate was fractionated in a distillation apparatus with a column 21 cm. in length under 0.2 mm. pressure as recorded in Table III.   Each fraction contained approximately 130 mg. of material except Fractions 1 and 20 which contained much less.

TABLE III

Fractionation of Hydrocarbons

Fraction No.	Bath temperature	Column temperature	n_D^{23}	Analysis	
				C	H
	°C.	°C.		per cent	per cent
1	170	110	1.5741		
2	172	125	1.5788	91 10	9 25
3	175	130	1 5792	90 57	9.36
4	179	135	1.5871	90.61	9.21
5	182	135	1 6005	91.06	8.96
6	187	138	1.6235	91.55	8.12
7	192	141	1.6403		
8	190	146	1 6481	92.69	7.70
9	186	150	1 6517		
10	185	150	1 6526	92.70	7.29
11	188	145	1.6536		
12	185	150	1 6548	92 64	7.50
13	186	147	1.6558		
14	190	150	1.6558	92.70	7.55
15	190	150	1 6520		
16	193	150	1.6528	92.85	7 48
17	200	160	1.6444		
18	210	170	1 6419	91.77	8 16
19	210	190			
20	250	220			

Fraction 2 crystallized directly in largest part and Fractions 9 to 15 crystallized on standing.   The analytical data in general agreed with the figures for the $C_{17}H_{16}$ hydrocarbon reported by Lawson and Topps (calculated, C 92.68, H 7.32).   Fractions 19 and 20 contained free selenium. Fraction 20 was found also to contain crystalline hydrocarbon material.

1-Methylphenanthrene—Fraction 2 after two recrystallizations from ether melted at 117–121°.

$C_{15}H_{12}$.   Calculated.   C 93.70, H 6.25, mol. wt. 192.1
                 Found.   " 93.37, " 6.49, "   " 195.2

Synthetic 1-methylphenanthrene (which melted at 122–124°) gave a mixture with the above hydrocarbon which melted at 119–121°.

8 mg. of the hydrocarbon yielded 12 mg. of picrate from ethyl alcohol as well formed, orange needles which melted at 137–139°.

$C_{15}H_{12} \cdot C_6H_3O_7N_3$.   Calculated, C 59.84, H 3.59; found, C 60.00, H 3.87

The picrate prepared from synthetic 1-methylphenanthrene melted at 138–140° and showed no depression when mixed with that from the above hydrocarbon.

The $C_{17}H_{16}$ Hydrocarbon—Fraction 14 was somewhat sticky at room temperature but did not melt until a temperature of 40° was reached. Upon recrystallization from isopentane it formed square plates which melted at 41–43°.

$C_{17}H_{16}$.   Calculated, C 92.68, H 7.32; found, C 92.61, H 7.30

The picrate crystallized from acetone as orange needles which melted at 129–131°.

$C_{17}H_{16} \cdot C_6H_3O_7N_3$.   Calculated, C 61.44, H 4.26; found, C 61.54, H 4.28

The earlier fractions also appeared to consist largely of this hydrocarbon. Although Fraction 6 could not be induced to crystallize directly, it formed in good yield a 1,3,5-trinitrobenzene derivative which after recrystallization melted at 145–148°.

$C_{17}H_{16} \cdot C_6H_3O_6N_3$.   Calculated, C 63.71, H 4.42; found, C 63.79, H 4.11

The $C_{18}H_{18}$ Hydrocarbon—The major part of Fraction 18 was treated with 70 mg. of 1,3,5-trinitrobenzene in acetone. The product which crystallized after recrystallization formed fuzzy needles which melted at 163–166°.

$C_{18}H_{18} \cdot C_6H_3O_6N_3$.   Calculated, C 64.40, H 4.73; found, C 64.58, H 4.50

The hydrocarbon recovered from the trinitrobenzene derivative by reduction with Zn and HCl formed an oil which was distilled under 0.2 mm. pressure. The distillate did not crystallize. It gave the correct analytical figures for $C_{18}H_{18}$.

$C_{18}H_{18}$.   Calculated, C 92.25, H 7.75; found, C 92.17, H 7.92

A portion of the oily hydrocarbon was treated with an equivalent of picric acid. After two recrystallizations from a mixture of acetone and ethyl alcohol, small orange needles of the picrate were obtained which melted at 153–156°.

$C_{18}H_{18} \cdot C_7H_3O_7N_3$.   Calculated, C 62.18, H 4.56; found, C 62.50, H 4.45

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THE ACONITE ALKALOIDS

IX. THE ISOLATION OF TWO NEW ALKALOIDS FROM ACONITUM HETEROPHYLLUM, HETERATISINE AND HETISINE

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In the course of the isolation of atisine from the roots of *Aconitum heterophyllum* of commercial origin, as presented in Paper VIII (1), it was noted that further concentration of the final mother liquors from atisine hydrochloride yielded a thick solution which on longer standing gave an additional crystalline fraction. It was then found that this fraction (10 gm. from 24 kilos of root) consisted essentially of a mixture of the hydrochlorides of two additional alkaloids. As far as it has been possible to ascertain, it appears that these alkaloids have not been previously described.[1] One of these formed the major portion of the above mixture and most of it was readily separated from its companion alkaloid by virtue of its sparing solubility in benzene. It crystallized very readily from alcohol as stout prisms which melted at 262–267° with decomposition and was optically active, $[\alpha]_D = +40°$ in methanol. The trivial name *heteratisine* is suggested for this alkaloid.

The analytical data all conformed to the formulation $C_{22}H_{33}O_5N$. The alkaloid contains one methoxyl group and an N-methyl group. No unsaturated linkage could be detected by hydrogenation. The presence of a lactone group was shown by its behavior towards alkali. On reacidification relactonization occurred. Aside from saponification of the lactone group the alkaloid did not appear to be affected by alkali. The presence of two hydroxyl groups was indicated by the Tschugaeff-Zerewitinoff determination. The *hydrochloride* of heteratisine was also prepared.

From the above data heteratisine, like atisine, appears to be pentacyclic and this fact along with the apparent occurrence of these alkaloids in the same plant makes it probable that they are derivatives of a common pentacyclic ring system. The formulation of heteratisine and its possession of a lactone group recall in some respects observations which have been recorded with alkaloids obtained from *Stemona* species; viz., the

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[1] Lawson and Topps (2) were of the opinion that, "Atisine appears to be the only base [alkaloid] in *A. heterophyllum*." The high yield of atisine which we have obtained is suggestive of the good quality of the commercial root with which we have worked and there appears to be no reason to believe that the new alkaloids owe their presence to a contaminating plant source. However, it is desirable to confirm our observations with botanically identified *Aconitum heterophyllum*.

stemonine $C_{22}H_{33}O_4N$ of Lobstein and Grumbach (3), the tuberostemonine $C_{22}H_{33}O_4N$ of Kondo, Suzuki, and Satomi (4), and the unnamed alkaloid $C_{22}H_{33}O_4N$ obtained from *Stemona sessilifolia* by Schild (5). In the last two cases the alkaloids were found to contain a lactone group and could be reduced, however, to dihydro derivatives, $C_{22}H_{35}O_4N$.

The second alkaloid, which was obtained in very small amount and which can be called *hetisine*, was separated from residual heteratisine most satisfactorily by chromatographic fractionation. It was more slowly eluted from the column than heteratisine and was isolated first as the *hydrochloride*. The base itself which crystallized readily from dilute alcohol melted at 253–256° and was also optically active, $[\alpha]_D = +13.7°$ in alcohol. The analytical data from both the alkaloid and its hydrochloride suggested a formulation of $C_{20}H_{27}O_3N$. It contained no methoxyl group and the N-methyl determination appeared also to exclude the presence of an N-methyl group.[2] The Tschugaeff-Zerewitinoff determination indicated the presence of 3 active H atoms. Whether all of these were due to three hydroxyl groups has not been directly determined. The alkaloid appeared to be unaffected by heating with alkali under conditions which should have permitted the detection of ester linkages. The presence of at least one double bond in hetisine was shown by hydrogenation of the hydrochloride to the *hydrochloride of dihydrohetisine*.

Our data with hetisine present a close analogy with those given recently for the new alkaloid kobusine, $C_{20}H_{27}O_2N$, described by Suginome and Shimanouti (6). The latter was shown to contain two hydroxyl groups, a tertiary N atom, and two unsaturated linkages. In the last respect a difference was shown, since hetisine appeared to yield only dihydrohetisine on hydrogenation. This is a point among others which will be studied further when opportunity is presented.

<div align="center">EXPERIMENTAL</div>

Heteratisine—The mother liquors from which the final crops of atisine hydrochloride had been obtained as described in the previous paper (1) were concentrated further to a syrup. When allowed to stand for several weeks an additional crop of crystals gradually formed. After collection with cold 95 per cent alcohol roughly 10 gm. were obtained from 24 kilos of atis root. This proved to be a mixture of alkaloid salts. 7.9 gm. of this mixture were decomposed in aqueous solution with excess alkali and the precipitated alkaloid was extracted with chloroform. The extract on concentration yielded a residue which when dissolved in benzene readily

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[2] The N(CH₃) determinations gave results, *viz.* 1.01 and 1.28, which were far too low for the calculated 4.1. While such results cannot be ignored, they may have been due to a C(CH₃) rather than an N(CH₃) group.

crystallized. After collection with benzene 3.2 gm. of the new alkaloid were obtained.

After recrystallization from 95 per cent alcohol it formed short, stout, often rhombic or trapezoidal prisms, which melted gradually at 262–267° to a colorless mass which slowly effervesced. The alkaloid is rather sparingly soluble in the usual organic solvents.

$$[\alpha]_D^{27} = +40° \ (c = 1.07 \text{ in methyl alcohol})$$

$C_{22}H_{33}O_5N$. Calculated. C 67.47, H 8.50, N 3.58, OCH_3 7.93, $N(CH_3)$ 3.84

Found. (a) " 67.57, " 8.40, OCH_3 8.14, $N(CH_3)$ 3.08

" (b) " 67.30, " 8.51, N 3.56

" (c) " 67.58, " 8.57

The molecular weight was determined in camphor by the Rast method. Molecular weight calculated, 391.27; found, 394.

The Tschugaeff-Zerewitinoff determination showed the presence of 2 active H atoms. Calculated, 2H 0.515; found at 25°, 0.521; at 95°, 0.532.

0.1 gm. of the alkaloid was boiled in a 10 per cent methyl alcoholic solution of NaOH for 1 hour. The colorless solution remained clear on dilution and yielded nothing on extraction with chloroform. However, when the solution was acidified with an excess of H_2SO_4 for relactonization and then again made alkaline, a turbidity followed by crystallization occurred. The crystalline base was extracted with chloroform. The extract gave a residue which crystallized from alcohol in the form characteristic of the alkaloid used. It is apparent that relactonization of a saponified lactone group occurred. The product melted gradually from 261–266° with slow effervescence.

$$[\alpha]_D^{27} = +40° \ (c = 1.00 \text{ in methyl alcohol})$$
Found. C 67.67, H 8.61

The hydrochloride prepared from this base separated from alcohol-ether as rhombic platelets, the melting point of which depended upon the rate of heating. It slowly melted with decomposition at 265–270° after preliminary darkening and softening above 255°.

For analysis it was dried at 110° and 2 mm.

$C_{22}H_{34}O_5NCl$. Calculated, C 61.72, H 8.01; found, C 61.38, H 7.91

The benzene mother liquor from which the above alkaloid had crystallized was brought to a volume of 50 cc. and chromatographed through 150 gm. of Al_2O_3 (Merck and Company, Inc.). An attempt was first made to elute with benzene but even after 550 cc., collected in 50 cc. fractions, had passed through, no appreciable material was eluted from the column. On changing to 1 per cent methanol in benzene again no appreciable effect was noted. 4 per cent methanol was then employed. This eluent soon removed a band from the column which consisted essentially of more of the

above alkaloid.   0.5 gm. of the latter crystallized readily from benzene in characteristic form.   It gradually melted at 262–265° with slow effervescence after preliminary softening.   Found, C 67.80, H 8.39.

Hetisine—On continued elution of the column with 4 per cent methanol in benzene no further sharp band was obtained but a very gradually diminishing amount of material continued to be eluted.   Following the above sharp band succeeding volumes of 50 cc. of eluent at first yielded about 70 mg. of solid residue and after about twenty additional fractions had been collected this dropped to about 30 mg.   When finally 10 per cent methanol was used, a sharp band was again eluted which was found to have practically exhausted the column.

Since the alkaloid in these fractions did not appear to crystallize directly, a number of succeeding fractions were combined for conversion into the hydrochlorides which were found to crystallize.   Thus Fractions 35 to 39 were combined.   The residue obtained on concentration was dissolved in a minimum of 95 per cent alcohol and converted into the HCl salt.   The latter, 0.1 gm., formed small flat needles or prisms which decomposed at 300° after preliminary sintering.

$$C_{20}H_{28}O_3NCl.\quad\text{Calculated.}\quad C\ 65.63,\ H\ 7.72,\ N\ 3.83,\ Cl\ 9.70$$
$$\text{Found.}\qquad\ ``\ 65.56,\ ``\ 7.37,\ ``\ 3.91$$

The combined Fractions 42 to 47 yielded 0.14 gm. of hydrochloride which melted with decomposition at 306–308° after preliminary softening. Found, C 65.55, H 7.46.

Finally Fractions 51 to 56 which consisted of the sharper band eluted with 10 per cent methanol in benzene gave 0.3 gm. of a hydrochloride which melted with decomposition at 307–308°.   Found, C 65.37, H 7.63, N 3.85, Cl 9.37.

The second and third of these hydrochlorides were combined for isolation of the free base.   On treatment of the aqueous solution with dilute NaOH a pasty mass precipitated.   When extraction with benzene was attempted, crystallization rapidly occurred and the material proved to be too insoluble in benzene.   Even a relatively large volume of chloroform was required to extract the alkaloid.   The concentrated chloroform extract was dissolved in a small volume of 95 per cent alcohol.   On careful dilution the alkaloid crystallized readily as broad flat needles or platelets which sintered above 245° and gradually melted at 253–256°.

$$[\alpha]_D^{25} = +13.7°\ (c = 1.02\ \text{in 95 per cent alcohol})$$
$$C_{20}H_{27}O_3N.\quad\text{Calculated.}\quad C\ 72.90,\ H\ 8.27,\ N\ 4.26$$
$$\text{Found.}\qquad\ ``\ 72.80,\ ``\ 8.04,\ ``\ 4.50;\ C\ 72.86,\ H\ 8.26$$

The sparing solubility of the alkaloid in camphor made unreliable the attempts at a molecular weight determination.   The active hydrogen de-

termination was as follows: 13.980 mg. of substance gave 3.05 cc. of CH_4 (25°, 734.5 mm.); at 95° there was no change. Found, 0.87; calculated for 3H, 0.918.

As a control a portion of the crystalline alkaloid was reconverted into the hydrochloride. This now melted with decomposition at 325° (uncorrected). Found, C 65.80, H 7.72.

The alkaloid did not appear to be affected by alkali. A solution of 0.15 gm. of the hydrochloride in 4 cc. of 10 per cent methyl alcoholic NaOH was refluxed for 1 hour. The colorless mixture after dilution was extracted with chloroform. The residue obtained after concentration of the extract was reconverted into the hydrochloride. This melted with decomposition at 315° (uncorrected). Found, C 65.52, H 7.72.

Dihydrohetisine—0.1 gm. of the hydrochloride of the above alkaloid was hydrogenated in methyl alcoholic solution with 50 mg. of platinum oxide catalyst under 3 atmospheres pressure. Absorption was prompt and practically completed within 10 to 15 minutes. After several hours the absorption of H_2 was about 8 cc. in excess of that required by the catalyst, calculated for 1 mole, about 6 cc. The product crystallized readily from alcohol-ether as needles which after recrystallization decomposed at 333° (uncorrected) after preliminary softening and darkening.

$C_{20}H_{30}O_3NCl$. Calculated, C 65.27, H 8.22; found, C 65.30, H 8.16

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THE ACONITE ALKALOIDS

X. ON NAPELLINE

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A few years ago (1937) Freudenberg and Rogers (1) reported the isolation of a new alkaloid, napelline, from the amorphous mixture of residual bases which had been obtained in the preparation of aconitine (Merck) from the roots of *Aconitum napellus*. Analyses of the alkaloid and a number of its salts indicated a formula $C_{22}H_{33}O_3N$ for the base. The only additional data presented since appear to be the brief announcement by these workers (2) of the isolation of a hydrocarbon $C_{17}H_{16}$ in dehydrogenation experiments. The identity of the product with the $C_{17}H_{16}$ hydrocarbon described by Lawson and Topps (3) as a dehydrogenation product of atisine was suggested. The hydrocarbon was characterized analytically only as the picrate (m.p. 130°), although the trinitrobenzene derivative (m.p. 138°) was also recorded.

In an effort to obtain a broader comparative basis for the suspected structural relationship of the simpler alkaloids of the atisine, staphisine, napelline, etc., group with the somewhat more complicated alkamines of the aconitine-delphinine group, we have recently extended our study to napelline. By following essentially the procedure of Freudenberg and Rogers the base was isolated as the hydrobromide. Analyses of different samples of this salt were in fair agreement with their formula of $C_{22}H_{33}O_3N \cdot$ HBr. An N-alkyl (presumably methyl) but no methoxyl group was found to be present. The Tschugaeff-Zerewitinoff determination on the sublimed base showed the presence of 3 active H atoms presumably due to three hydroxyl groups. On hydrogenation of the hydrobromide, approximately 1 mole of H_2 was absorbed, and analysis of the resulting salt supported the formulation $C_{22}H_{35}O_3N \cdot HBr$ of a *dihydronapelline hydrobromide*. This was undoubtedly a mixture of isomers. A relatively small fraction of the liberated base crystallized from ether but exhibited an indefinite melting point. The analytical results obtained with the crystalline base, however, were not in agreement with the calculated figures, since the H results were too low. However, on reconversion to the HBr salt the analytical results again approached the requirements of a dihydro derivative. No explanation for this discrepancy has been found.

On the basis of the absorption of hydrogen only one double bond has

been directly shown in the molecule and because of the presence of three hydroxyl groups the alkaloid would appear to be hexacyclic. However, atisine has been shown with some certainty to be of pentacyclic nature. It is quite possible that napelline also is pentacyclic and possesses a second double bond which is very difficult to hydrogenate.

We have attempted a repetition of the study of the dehydrogenation of napelline which was very briefly reported by Freudenberg and Rogers. The main product was a mixture of hydrocarbons which, however, with the amount available, proved difficult to separate by fractionation alone. One of the first fractions did not crystallize directly but yielded a picrate which melted at 142–146° and from the analysis appeared to be the picrate of a dimethylphenanthrene (or ethylphenanthrene).

The hydrocarbon produced in relatively largest amount melted at 76–79°. The analysis of the latter and of its derivatives agreed with the formulation $C_{18}H_{18}$.[1] The ultraviolet absorption spectrum curve, as given in Fig. 1, strongly points to an alkylphenanthrene, perhaps a tetramethylphenanthrene. It formed a picrate which melted at 132–134° and a trinitrobenzene derivative which melted at 150–153°.

We have been unable, however, to confirm the formation of the hydrocarbon $C_{17}H_{16}$ such as reported by Freudenberg and Rogers, which they suggested to be identical with the characteristic atisine dehydrogenation hydrocarbon. The melting point 132–134° of our picrate is close to that of 130° reported by these workers for their picrate. It is possible they had in hand the $C_{18}H_{18}$ picrate. The lower melting point of 138° given by them for their trinitrobenzene derivative as against the above melting point of 150–153° could have been due to contamination.

An appreciable crude basic fraction was also produced during the dehydrogenation. From this, however, after preliminary rough fractionation a crystalline picrate was obtained in very small yield. Analysis of this suggested a formulation $C_{17}H_{17}N$ for the base. The melting point of the picrate, 233–237°, was higher than that of 206° recorded by Lawson and Topps for the picrate of the $C_{17}H_{17}N$ base described by them as a product from atisine.

<div align="center">EXPERIMENTAL</div>

Napelline—The alkaloid was isolated from amorphous aconitine (Merck and Company, Inc.) essentially as described by Freudenberg and Rogers (1). The hydrobromide agreed closely in properties with those recorded by them. The melting point, or rather decomposition point, varied somewhat with individual fractions. This was usually 227–230° after pre-

[1] The absorption spectra curves were very kindly determined by Dr. George I. Lavin of The Rockefeller Institute for Medical Research.

liminary softening and discoloration, although some fractions decomposed at 237–240°.

FIG. 1. Absorption spectrum curves in ethyl alcohol solution. ● = the $C_{18}H_{18}$ hydrocarbon from napelline; the solid line = phenanthrene.

For analysis it was dried at 110° and 2 mm.

$C_{22}H_{32}O_3N \cdot HBr$. Calculated. C 59.97, H 7.78, N(CH$_3$) 3.41
                          Found. (a)   " 59.57, " 7.60
                          "   (b)   " 59.81, " 7.60
                          "   (c)   " 59.69, " 7.57
                          "   (d)   " 59.97, " 7.53
                          "   (e)   " 59.41, " 7.53, N(CH$_3$) 2.74

The methoxyl determination was negative.
The free base was liberated from a sample of the salt with 10 per cent

NaOH and extracted with warm benzene. The extract after drying over K_2CO_3 was concentrated to dryness in a molecular still. The free base distilled at a bath temperature of 140–150° and 0.1 μ. The solid distillate, which was possibly amorphous, melted at 85–88°. The molecular weight was determined in camphor.

$C_{22}H_{33}O_3N$. Calculated. C 73.48, H 9.26, mol. wt. 359.27
Found.   " 73.42, " 9.12, "   " 346.7

The Tschugaeff-Zerewitinoff active H determination was as follows: 10.455 mg. of substance gave 2.32 cc. of CH_4 (27°, 731.5 mm.); at 95° there was no change. Found, H 0.876; calculated, 3H 0.841.

Dihydronapelline—0.2 gm. of napelline hydrobromide was hydrogenated in methyl alcoholic solution with 50 mg. of platinum oxide catalyst under about 3 atmospheres pressure. About 10 cc. of H_2 was absorbed by the substance, or roughly 1 mole. The concentrated solution on addition of ether yielded delicate needles which melted at 256–258° after preliminary sintering.

$C_{22}H_{35}O_3N \cdot HBr$. Calculated. C 59.70, H 8.20
Found. (a) " 59.70, " 8.02; (b) C 59.42, H 8.06

The free base was obtained after decomposition of the salt with 10 per cent NaOH and extraction with ether. The extract after drying over K_2CO_3 yielded from the concentrated solution delicate needles which gave a micro melting point of 145–160° with a few crystals persisting up to 165°. The major portion remained dissolved in the mother liquor. A mixture of substances was obviously produced and with the amount of material available no attempt at further separation could be made. The analytical results obtained with the crystalline material are difficult to explain. The hydrogen figures from several experiments were too low.

$C_{22}H_{35}O_3N$. Calculated. C 73.07, H 9.76
Found. (a) " 72.71, " 9.22; (b) C 72.83, H 9.30

The crystalline base was reconverted into the hydrobromide. The latter formed woolly masses of needles which, however, now gradually softened to a resin at 226–230°. Found, C 59.28, H 8.14.

Dehydrogenation of Napelline—15 gm. of napelline hydrobromide were shaken with 10 per cent NaOH and sufficient ether to extract the liberated base. The ether extract was dried over K_2CO_3 and evaporated to dryness under reduced pressure. 11 gm. of the resulting resin were ground with 35 gm. of selenium and after the air had been replaced by nitrogen, the mixture was heated at 340° for 2 hours.

The volatile material which condensed in the ice trap was mixed with ether and the solution was shaken with a slight excess of acid. The dried ether layer after evaporation gave approximately 0.34 gm. of oil which

could be distilled but contained selenium. It did not yield crystalline material. The acid aqueous layer was made strongly alkaline with NaOH and then extracted with ether. The aqueous alkaline layer contained no phenolic material. The ether layer yielded 0.1 gm. of oily residue which possessed the odor of pyridine bases but gave only a small amount of oil upon distillation. This distillate was unstable and did not yield to further investigation.

The undistilled residue from the dehydrogenation mixture was finely pulverized and exhaustively extracted with ether. The ether extract was shaken with 30 cc. of 10 per cent HCl. The acid layer contained a suspension of sticky tar which was readily extracted with chloroform and was evidently composed of mixed hydrochlorides of basic material. The

TABLE I

Fractionation of Hydrocarbons

Fraction No.	Bath temperature	Column temperature	Weight	Analysis	
				C	H
	°C.	°C.	mg.	per cent	per cent
1	180	130	40	90.26	9.64
2	185	135	120	90.79	9.19
3	185	145	120	91.67	8.30
4	185	155	120	91.86	8.07
5	185	165	120	92.20	7.89
6	185	168	120	91.81	8.30
7	195	164	120	91 87	8 23
8	195	164	120	89.74	8.15
9	215	170	120	91.66	8.18
10	230	185	120	89.28	8.48
11	230	220	120		
12	230	220	30		

aqueous layer which remained after extraction with chloroform contained practically no further salts of basic compounds. The chloroform solution was shaken with sodium hydroxide in order to yield a solution of free bases. After concentration to dryness in a sublimation apparatus, 1.3 gm. of a mixture were obtained. On fractionation 0.28 gm. of viscous resin distilled up to a bath temperature of 200° under 0.2 mm. pressure. This sublimate was placed in a microdistillation flask under 0.2 mm. pressure and again distilled. 0.16 gm. of viscous oil distilled up to 200°. Since it could not be crystallized as such, it was finally treated with 0.15 gm. of picric acid. 30 mg. of a picrate crystallized from acetone and after recrystallization the micro melting point was 233–237°.

$C_{17}H_{17}N \cdot C_6H_3O_7N_3$. Calculated, C 59.46, H 4.34; found, C 59.27, H 4.10

The above ether extract from the original dehydrogenation melt which

remained after extraction with HCl was dried over K_2CO_3 and evaporated to dryness. The residue weighed 2.95 gm. This was dissolved in 50 cc. of benzene and chromatographed through 250 gm. of Brockmann's alumina in benzene. 1.8 gm. of the material were not retained by the adsorbent and passed through the chromatograph with the original solvent.

This material was placed in a sublimation apparatus under 0.2 mm. pressure and all was collected which sublimed up to a bath temperature of 200°. The sublimate amounted to 1.5 gm. It was a viscous oil which did not crystallize. It was fractionated in a micro fractionating apparatus with a 22 cm. column at 0.2 mm. pressure, as recorded in Table I.

All of the fractions were of hydrocarbon character, as shown by the analytical data. Fractions 11 and 12 contained free selenium. Fraction 1 did not crystallize as such or as a picrate.

Fraction 2 did not crystallize directly but when treated with 50 mg. of picric acid in alcohol, 47 mg. of reddish brown needles of the picrate were obtained which after recrystallization showed a micro melting point of 142–146°.

$C_{16}H_{14} \cdot C_6H_3O_7N_4$. Calculated, C 60.66, H 3.93; found, C 60.79, H 3.86

Fractions 5 to 8 crystallized on standing and careful examination showed them to consist chiefly of a $C_{18}H_{18}$ hydrocarbon. Fraction 5 gave satisfactory analytical data for this substance without further purification. However, it did not possess a sharp melting point. After repeated recrystallization from ether, it formed rosettes of well formed columns which showed a micro melting point of 76–79°.

$C_{18}H_{18}$. Calculated, C 92.25, H 7.75; found, C 92.37, H 7.74
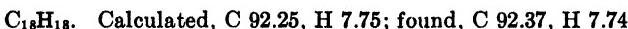
The hydrocarbon with an equivalent of picric acid yielded a picrate from a mixture of acetone and ethyl alcohol. After recrystallization it formed orange needles the micro melting point of which was 132–134°.

$C_{18}H_{18} \cdot C_6H_3O_7N$. Calculated, C 62.18, H 4.56; found, C 62.46, H 4.51
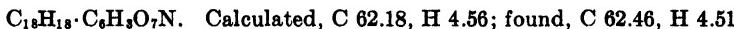
The 1,3,5-trinitrobenzene derivative crystallized from acetone and after recrystallization formed rosettes of yellow needles which melted at 150–153° (micro melting point).

$C_{18}H_{18} \cdot C_6H_3O_6N_3$. Calculated, C 64.40, H 4.73; found, C 64.23, H 4.71
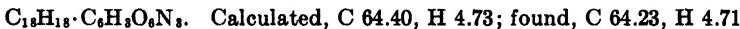
The hydrocarbon recovered from both the picrate and trinitrobenzene derivative did not differ in properties from the original hydrocarbon purified by direct recrystallization.

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CYCLIC VARIATIONS IN THE LIPIDS OF THE CORPUS LUTEUM*

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The concept that the phospholipid content of a tissue is a function of its metabolic activity, first suggested by Mayer and Schaeffer (1), received its greatest support from the work of Bloor and his associates on the corpus luteum (2), mammary gland (3), tumors (4, 5), and muscles (6–8). Other workers, however, were unable to demonstrate such a relationship (9–12). The effect of metabolic activity on the cholesterol content has been studied less extensively. The results of Bloor (7) indicate that increase in activity of muscles is associated with lowered cholesterol levels.

The fact that the corpus luteum undergoes a cycle of development and, in the absence of impregnation, retrogression makes this organ ideal for a study of the relationship between lipid content and physiological activity. The present work was undertaken with this purpose in mind.

Early chemical studies of Chauffard, Laroche, and Grigaut (13), Rosenbloom (14), Fenger (15), and Corner (16) demonstrated the presence in the corpus luteum of large amounts of phospholipid and smaller, though variable, quantities of cholesterol, fats, and cholesterol esters. Kaufmann and Raeth (17) made lipid analyses of twenty-four human corpora lutea. At the height of function, namely in the "bloom" stage or during pregnancy, they reported that the lipids were not decreased, as early histological evidence indicated; there was an actual increase in the "lecithin." Bloor, Okey, and Corner (2) in a chemical and histological investigation of the corpus luteum of the sow found a marked variation in the phospholipid content with activity of the gland, and a similar, though less pronounced, variation in the free cholesterol. Cholesterol esters varied inversely with activity of the gland.

EXPERIMENTAL

The corpora lutea for this study were obtained from operations performed by members of the gynecology staff of St. Luke's Hospital of Chicago. The organ, dissected from the ovary in the operating room, was separated

in the laboratory into two approximately equal parts, one for cytological and one for chemical study. The portion for chemical study was weighed, ground without sand in a mortar, and transferred to a weighed paper thimble. The tissue was extracted with absolute alcohol for 12 hours in a Soxhlet apparatus, then for 4 hours with ether. The combined extracts were evaporated and the residue dissolved in 25 ml. of petroleum ether. Weighing of the thimble after extraction gave the weight of the non-lipid residue. This procedure allowed the calculation of the moisture content as the difference between the moist weight of the tissue and the sum of the lipid extract and the non-lipid residue.

10 to 25 ml. aliquots of the petroleum ether extract were used for the lipid analyses. When material permitted, they were carried out in duplicate. The phospholipids were precipitated by the method of Bloor (18), separated into ether-soluble and ether-insoluble fractions, and lecithin, cephalin, and sphingomyelin were determined according to Kirk (19), except that phosphorus estimations were carried out by a photoelectric modification of the method of Fiske and Subbarow (20). Cholesterol, free and total, was determined on suitable aliquots of the acetone-soluble fraction by the method of Schoenheimer and Sperry (21). The glyceride fraction was estimated by oxidation of the acetone-soluble lipids and subtraction of the cholesterol equivalents.[1]

Results

The values obtained for the lipids of the human corpus luteum are in Table I. The specimens are arranged for convenience in five age groups designated according to convention as young (proliferating), vascularizing, early and late bloom, and regressing. Values for the corpora lutea of pregnancy, which varied in age from $2\frac{1}{2}$ to 4 months, also are included. The corresponding age range, counted from the day of ovulation, is in the adjoining column. As the variations in moisture content in each group or between the groups are slight, no changes worthy of note would be produced by calculating the lipids on the basis of dry weight.

Free Cholesterol—Throughout the periods of growth and development

[1] In following the original procedure of Bloor (22) we experienced considerable difficulty in getting reproducible factors on the pure lipids. This was traced to unevenness of temperature in the oven and to the use of silver, which greatly decreased the stability of the dichromate. The method was modified by elimination of the silver and by heating the tightly stoppered flasks for a half hour in an autoclave at 18 pounds pressure (124°). Loss of dichromate in this procedure was negligible, even when the time of autoclaving was doubled. The oxidation factors for the pure substances were somewhat low: 3.48, 3.62, and 3.71 compared with theoretical values of 3.60, 3.76, and 3.92 ml. of 0.1 N dichromate per mg. of stearic acid, cholesterol stearate, and cholesterol respectively; but they were consistent and reproducible.

TABLE I

Corpus Luteum Lipids in Per Cent of Moist Weight of Tissue

Stage of development	Age	No. of specimens	Cholesterol, free total esters			Phospholipids	Glycerides	Total lipids	Moisture
	days								
Young	1– 3	6	0.21 (0.15–0.29)	0.41 (0.35–0.46)	0.34 (0.18–0.50)	1.32 (1.00–1.61)	1.05 (0.75–1.32)	2.92 (2.09–3.15)	83.8 (81.2–87.2)
Vascularization	5– 8	6	0.16 (0.10–0.26)	0.33 (0.22–0.45)	0.29 (0.19–0.44)	1.66 (1.38–2.03)	0.88 (0.63–1.41)	2.99 (2.56–3.54)	86.5 (85.2–88.7)
Early bloom	8–11	6	0.19 (0.14–0.24)	0.33 (0.29–0.38)	0.25 (0.11–0.30)	2.04 (1.78–2.82)	0.87 (0.51–1.11)	3.36 (2.78–4.25)	85.5 (82.0–87.6)
Late bloom	11–13	6	0.19 (0.15–0.24)	0.63 (0.45–0.74)	0.73 (0.42–0.92)	1.96 (1.38–2.81)	0.94 (0–2.19)	3.82 (2.40–5.65)	
Regression	14–18	8	0.33 (0.15–0.67)	1.07 (0.41–2.28)	1.25 (0.32–2.71)	1.71 (0.83–2.30)	1.79 (0.83–2.90)	4.85 (2.70–7.00)	84.1 (81.9–86.2)
Pregnancy	2½–4½ mos.	5	0.20 (0.13–0.31)	0.27 (0.17–0.42)	0.12 (0.05–0.19)	2.64 (2.24–3.31)	0.77 (0.54–1.41)	3.63 (2.93–4.93)	82.4 (78.5–87.9)

The numbers in parentheses are minimum and maximum values.

and during pregnancy the free cholesterol remained fairly constant at about 0.2 per cent of the fresh, moist tissue. Only during the regressing phase is the average value increased to 0.33 per cent. This value is somewhat misleading because, of the eight corpora lutea of this group examined, two gave free cholesterol values of 0.59 and 0.67 per cent respectively. If these two are disregarded, the average of the remaining six, 0.22 per cent, is not significantly different from those of the other groups.

Cholesterol Esters—During the first 10 days there is a slight decline in cholesterol esters, but beginning in the late bloom period, there is a striking increase which reaches its maximum during regression. Minimum values for cholesterol esters were observed in the corpora lutea of pregnancy. Though the average values decline during the developmental stages, there is a wide range of variation with considerable overlapping. A significant decrease was observed only in the actively functioning corpus luteum of pregnancy.

TABLE II

Lecithin, Cephalin, and Sphingomyelin Content of Corpora Lutea in Per Cent of Total Phospholipid

State of development	Lecithin	Cephalin	Sphingo-myelin
Young....	52.0	38.2	9.8
Vascular......	48.2	33.1	18.7
Bloom	49.2	41.2	9.5
Regression.....	46.7	39.2	14.1
Pregnancy........	49.2	41.3	9.5

Phospholipid—A gradual, steady increase in phospholipid occurs with age of the gland, reaching a maximum of about 2 per cent during the bloom period and declining thereafter. Even during regression, the average, 1.7 per cent, is somewhat higher than the 1.3 per cent in the stage of proliferation. The consistently higher phospholipid values for the corpus luteum of pregnancy average 2.64 per cent. The average phospholipid values found here are considerably lower than those reported by Bloor, Okey, and Corner (2) for the corpus luteum of the sow, but are higher than the figures of Kaufmann and Raeth (17) for human corpora lutea. The values obtained by the latter authors are low, probably because they dried the tissues at 100° before extraction, a procedure now known to result in low phospholipid recovery. All three studies, nevertheless, concur in the finding of higher phospholipid values during periods of active function.

Phospholipid Partition—As shown in Table II, the proportions of the three types of phospholipid remain essentially constant in spite of marked

changes in the total phospholipid. The lecithin proportion is highest in the young corpora lutea at 52 per cent and lowest during regression, but the slight differences are not significant because of the variation in the proportions of individual phospholipids in each group. On the whole, approximately 50 per cent lecithin, 40 per cent cephalin, and about 10 per cent sphingomyelin constitute the corpus luteum phospholipid, the proportion remaining about the same regardless of the stage of development of the corpus luteum. The distribution pattern for the individual phospholipids resembles that found by Thannhauser and coworkers (23) for lung, spleen, kidney, liver, and heart, despite differences in the methods of analysis.

Glycerides—Glycerides, like the cholesterol esters, are relatively constant during developmental and functional periods, showing a rise only during the regressive phase. As this is the least accurately determined lipid, and as individual variations are great, their relation to activity of the gland is inconclusive.

DISCUSSION

Of the lipids studied, the most marked changes were observed in the cholesterol esters and phospholipids. Their relation to the functional activity of the human corpus luteum is clear. As the organ develops after ovulation, the phospholipids increase steadily, whereas the cholesterol esters remain constant or decrease slightly. If impregnation of the ovum occurs, functional activity continues at a high level; the corpus luteum of pregnancy is characterized by maximum phospholipid and minimum cholesterol esters. If, however, fertilization does not ensue, the gland has fulfilled its function, and at about the 10th day of its life, degeneration begins, with an abrupt rise in cholesterol esters and a simultaneous gradual decrease in phospholipid.

In discussions of the relation of lipids to physiological activity, significance has been attached to the ratio of phospholipid to cholesterol (24). This study indicates that changes in this ratio are only reflections of changes in the phospholipids, the cholesterol remaining relatively constant in spite of wide variations in functional activity of the gland. Only during degeneration, when cholesterol ester is high, is there a notable decrease in the ratio of phospholipid to total cholesterol. Similarly, the relation of total cholesterol to free cholesterol undergoes no marked variation with activity, though it is somewhat lower during periods of active function. Changes observed in the ratio are due solely to changes in the combined cholesterol.

The origin of the excess cholesterol esters during the regressive phase is uncertain. Bloor, Okey, and Corner remarked that the increase in concentration of total cholesterol in the degenerating corpus luteum of the

sow may be explained solely by the decrease in size of the gland during this period; according to this study, however, a 3-fold rise in total cholesterol is already evident in the late bloom period, when no shrinkage of the gland is obvious.

Sinclair (25), on the basis of differences in the activity of phospholipids from different parts of the body, has suggested that probably two types of phospholipid exist: the active metabolic, which has a high rate of fatty acid turnover, and the inactive, whose rôle is structural rather than metabolic. If such differences exist in the respective functions of the lecithin, cephalin, and sphingomyelin, variations would be expected in their proportions according to the activity of the tissue.

Although the partition method is only approximate, the lack of significant differences in the relative proportions of the three phospholipids with variations in the activity of the corpus luteum suggests that metabolic and structural functions of the phospholipids are not connected with the known chemical differences between them.

SUMMARY

Free cholesterol remains relatively constant throughout all stages of development of the human corpus luteum. The cholesterol esters remain constant or decline slightly with increasing activity, reaching their lowest values in the actively functioning corpus luteum of pregnancy. In the regressive phase, cholesterol esters increase to about 5 times their value during functional periods.

Phospholipids increase gradually with age of the organ, reaching maximum values during maximum function. They decline slightly during regression.

The glycerides do not vary appreciably in the developmental stages of the organ. A large increase is observed during regression.

The cyclic variation in the phospholipids is not accompanied by significant changes in the relative proportions of lecithin, cephalin, and sphingomyelin.

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THE BLOOD LACTATE-PYRUVATE RELATION AND ITS USE IN EXPERIMENTAL THIAMINE DEFICIENCY IN PIGEONS

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Pyruvic acid occupies an important position in the chain of reactions constituting carbohydrate breakdown in animal tissues. Not only does it appear to be the immediate precursor of lactic acid, but it is probably the substance immediately preceding the further breakdown of the 3-carbon intermediates (1).

Of the various pathways postulated by which pyruvic acid may be metabolized, all appear to involve the use of thiamine diphosphate (cocarboxylase) (2). The actual level of pyruvate in the tissues, however, is dependent on many other factors. Thus it has been recognized that, in addition to thiamine deficiency, even mild degrees of exercise (3) and glucose ingestion (4) cause increases of blood pyruvate. It is, therefore, hazardous to depend solely on a blood pyruvate estimation for detection of a thiamine deficiency. These facts combined with inadequate analytical methods have contributed to the confusion as to the value of blood pyruvic acid analysis for the detection of thiamine deficiency (5–8).

It has been known for some time that exercise (9) and anoxia (10, 11) give rise to increases in blood lactate.

As a result of blood studies preceding and following the exercise and anoxia of electric shock convulsions, a rather close relation was noted between the blood pyruvate and lactate levels. Such a relation also existed in various states of exercise, excitement, anoxia, and food consumption in spite of wide fluctuations of the absolute lactate and pyruvate levels. This would indicate that the equilibrium between these two substances is quite rapidly established, although a recent note by Friedemann and Barborka (12) indicates that, even after mild exercise, a definite period of 5 to 6 minutes may be required to establish normal lactate-pyruvate relations.

With the use of such a relation, it was possible to examine more closely the effect of thiamine deficiency on pyruvate metabolism as reflected in the blood, in spite of fluctuations of the absolute levels produced by difficultly controlled factors of experimentation. A marked change in the blood lactate-pyruvate relationship coincident with thiamine deficiency

has been noted in pigeons, in fact a correlation with the degree of deficiency. Thus in spite of the apparent close relation between lactate and pyruvate under most conditions, the decreased removal of pyruvate in thiamine deficiency leads to establishment of an abnormal lactate-pyruvate relation. The use of the relation is of particular value in experimental animals or subjects in which a controlled state of activity is difficult to maintain.

The object of this paper is to present proof of the constancy of the lactate-pyruvate relations under various conditions in humans, rats, and pigeons, and to illustrate the changes in this relation during controlled acute and chronic thiamine deficiency in pigeons.

EXPERIMENTAL

Blood was collected from the brachial veins of humans, stasis being avoided, and oxalate-iodoacetate was employed as recommended by Bueding and Wortis (13). Rat blood was collected by heart puncture or severing of the neck, with collection directly into oxalate-iodoacetate. Pigeon blood (0.6 cc.) was collected from the wing vein. A 1:5 trichloroacetic acid filtrate of the bloods (1:8 for pigeon blood) was prepared within 5 minutes of blood collection.

Pyruvic acid analyses were run on 3 cc. samples of the filtrate by the 2,4-dinitrophenylhydrazone procedure described by Bueding and Wortis (13). The final color was determined with a Coleman DM spectrophotometer at $\lambda = 440$ mμ; a standard curve prepared with sodium pyruvate standardized by bisulfite-iodine was employed.

Lactic acid determinations were performed on the appropriate dilutions of the trichloroacetic filtrates by the p-hydroxydiphenyl reaction employed by Miller and Muntz (14) and the modification of Koenemann (15). The modifications introduced by Barker and Summerson (16) involving copper lime treatment of the filtrates for removal of glucose and addition of Cu^{++} to increase the sensitivity of the color reaction were introduced only after this work was completed. We have since compared several bloods by this method and the older method employed in this work, and find that our lactic acid results may be 4 to 7 per cent too high. Although the newer method is to be recommended, the use of the older method involves a negligible correction of the results here reported and does not change their interpretation.

Lactate-Pyruvate Relations in Humans and in the Rat

The data collected from experimentation with humans were on normal, depressed, and schizophrenic subjects, no difference being noted between the groups. The values above 60 mg. per cent of lactic acid were in gen-

eral obtained in patients 5 to 10 minutes after electric shock convulsions, in which a condition of exercise and anoxia both play a rôle in elevating the blood lactate. In some cases, successive samples were taken on the same individual during the period following the convulsion. The lower values were obtained on both normal and psychotic patients at various degrees of rest, following mild exercise, and following consumption of glucose or ordinary meals. Since under these various conditions a fixed relation was found between lactate and pyruvate, all the data have been included in a single graph (see Fig. 1). Our object in this paper has been to include a large number of individuals (58 subjects) under the varied conditions
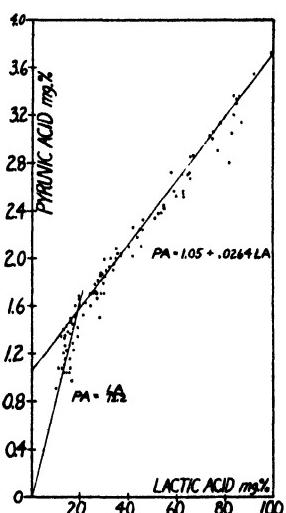
FIG. 1

FIG. 2

FIG. 1. Blood lactate-pyruvate relations in humans.
FIG. 2. Blood lactate-pyruvate relations in rats.
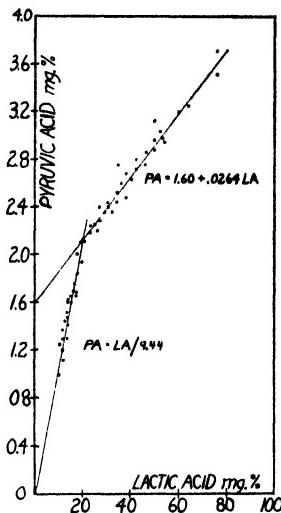
likely to be encountered rather than to make an intensive study of a few subjects.

The data collected on rats were on normal animals under various conditions of rest. In these animals, a controlled state of rest is more difficult to attain than in humans. Some of the lower lactate values were obtained only after cord resection, the higher ones after exercise and mild degrees of anoxia. The results are plotted in Fig. 2.

An examination of Figs. 1 and 2 shows that, in spite of individual variations and the varied experimental conditions referred to, there is a definite relation between the blood lactate and pyruvate levels. Above a blood lactic acid of 20 mg. per cent, the points fall on a line which would, in the

case of humans, intercept the axis at 1.05 mg. per cent of pyruvic acid, in the case of the rats, at 1.6 mg. per cent. Below 20 mg. per cent of lactic acid, the points fall on a line extending to the origin. It is possible that above 20 mg. per cent of lactic acid the muscles determine in large part the lactate-pyruvate relations, and below 20 mg. per cent, the other tissues.

TABLE I

Blood Pyruvic Acid Excesses (PA_{excess}) in Pigeons during Thiamine Deficiency

$PA_{excess} = PA_{found} - PA_{calc}$. The values are given in mg. per cent.

Thiamine daily during chronic stage	Bird No.	Normal; Mar. 11-15, 1941	9 days, no thiamine (acute); Mar. 26	14 days, no thiamine (acute); Mar. 31	12 days on low thiamine (chronic); Apr 12	25 days on low thiamine (chronic); Apr. 25	4 days after repair with 100 γ thiamine daily; Apr. 29	11 days after repair with 100 γ thiamine daily; May 6
γ 20	52	−0.14		4.84*	5.02	7.36	0.23	0.14
	67					5.69		
	54	−0.29†		1.21	2.87‡	5.94	0.08	0.30
	62	0.12	1.07†	2.82§	2.77‡	3.45‡	0.60	0.30
	59	−0.08, 0.06	2.51	4.16				
	75	0.20	1.66	1.85§	6.92†	5.71	0.70	0.36
	63	0.29†	2.85	2.81	4.29	5.54	1.30	2.00
25	58	−0.02, −0.26†		0.38§	2.44	4.97	0.22	0.15
	57	0.09	2.53 •	2.32	1.55	1.27	0.90	−0.21
	72	0.0		5.14*	2.66	2.15	0.57	1.40
	56			1.64§	3.00	2.35	0.79	0.53
	73	−0.13, 0.05†	1.61†	3.70*§	2.38	3.40	0.57	0.13
	68	0.07†	2.71					
	51	0.21	2.25					
	69		2.06†					
	74		1.05					

* In mild opisthotonus.
† Exercised.
‡ In noticeably better condition than other birds in the same group.
§ Repaired the day before with 20 γ of thiamine.

Since points falling anywhere on these two straight lines must be considered "normal" for the various states of activity encountered, only points removed from the lines can be considered indicative of more fundamental changes in the metabolism of lactate or pyruvate.

In a consideration of thiamine deficiency it is convenient to express these relations as pyruvic acid values. Because the lines representing the lactate-pyruvate relation have different slopes, two expressions must be

formulated. If we let LA equal the lactic acid value found, then for humans at LA <20 mg. per cent, $PA_{calc.} = LA/12.2$; at LA >20 mg. per cent, $PA_{calc.} = 1.05 + 0.0264LA$. For rats at LA <20 mg. per cent, $PA_{calc.} = LA/9.44$; at LA >20 mg. per cent, $PA_{calc.} = 1.60 + 0.0264LA$.

$PA_{calc.}$ is then the pyruvic acid value calculated from the normal relations of pyruvic and lactic acid illustrated in the graphs. The figures 1.05 (in humans) and 1.60 (in rats) represent the points of intercept of the lines with the pyruvic acid axis, and the figure 0.0264 the slope of the line.

In normal cases the pyruvic acid value calculated from the above formulas should be the same as the value found experimentally; that is, $PA_{calc.} = PA_{found}$. In thiamine-deficient subjects, the difference between the pyruvic acid value found and that calculated from the normal relations (pyruvic acid excess) might represent the degree of thiamine deficiency; that is, $PA_{excess} = PA_{found} - PA_{calc.}$.

Such expressions have been valuable in assigning a single figure for the degree of disturbance of pyruvate metabolism in the thiamine-deficient pigeons.

Thiamine Deficiency in Pigeons

Blood lactate and pyruvate studies were made on a group of twelve pigeons on the usual grain diet (17). Some were exercised to observe a wider range of lactate and pyruvate values. So called resting lactic acid values ranged from 23 to 45 mg. per cent and upon exercise could be elevated to as much as 130 mg. per cent. All of the lactate-pyruvate values fell on a line with an intercept of 2.0 on the pyruvic acid axis. In the case of normal pigeons $PA_{calc.} = 2.0 + 0.0135LA$. Thus the slope of the line in the case of pigeons is only about one-half that found in the case of humans and rats. We have not presented a plot of the data in the case of pigeons but have summarized the PA_{excess} values in the normal birds and in various states of thiamine deficiency in Table I. In the normal birds the PA_{excess} values fall from −0.29 to +0.29 mg. per cent.

The birds were then placed on a thiamine-free diet and tube-fed to assure uniform caloric intake, in a manner previously described in publications from this laboratory (17). After 9 days on this diet, blood studies were again made. It is significant that at this time no symptoms other than occasional regurgitation of food immediately after feeding could be observed in the birds. Nevertheless examination of Table I reveals that large increases in the PA_{excess} values may be of use even in detecting mild degrees of thiamine deficiency.

The birds were continued on the same diet for 5 more days or a total of 14 days on the thiamine-free diet, after which time mild regurgitation of food was common. On the 13th day some of the birds already showed

opisthotonus and were given a dose of 20 γ of thiamine intramuscularly for temporary repair. On the 14th day, blood studies were repeated. During the blood collection, three of the birds (noted in Table I) were in mild opisthotonus. These had the highest PA_{excess} values (3.70 to 5.14). Some of the lowest values occurred in those birds which had received the repairing dose of thiamine the previous day. In general the values were considerably higher than those found at the 9 day stage.

The birds were then divided into two groups, one of which received 20 γ of thiamine daily (intramuscularly) and the other 25 γ daily, all being maintained on the same forced feed régime.[1] After 12 days on this schedule, blood lactates and pyruvates were again determined. With the exception of two birds in which one of us (O. A B.) had independently noted a less severe deficiency from clinical signs during the acute stage, the birds on the 20 γ level showed definitely higher PA_{excess} values than those on the 25 γ level (average of 5.5 and 2.4 respectively). Likewise after 25 days on the thiamine-low diets, a real difference (now with only one exception) was noted in the PA_{excess} values of the two groups (average of 6.1 and 2.8 respectively). Of importance was the fact that such a separation of the groups on the basis of the PA_{excess} values was not as evident from the pyruvic acid levels alone. This was particularly true, since, as a result of leg weakness developed by a few birds before regular therapy was instituted, some birds remained in a comparatively quiet state, while others struggled vigorously to adopt a standing position, resulting in great variations in the actual lactate and pyruvate levels. Since a distinct difference in PA_{excess} values can be noted between birds receiving 25 γ of thiamine daily and those receiving only 20 per cent less, the use of PA_{excess} values may be useful in determining relatively small differences in degrees of thiamine deficiency in other animals.

The ten birds remaining in the group were then given 100 γ of thiamine daily and blood studies made 4 days later. It was surprising to find that the normal PA_{excess} of zero was reestablished in only three of the birds, although all showed a decrease. Even after 11 days of thiamine therapy, two of the birds still showed abnormal PA_{excess} values. Thus it would appear that even the chemical pathology of chronic thiamine deficiency, not to mention the anatomical, may be slow in returning to normal.

SUMMARY

It has been found that in a variety of conditions such as excitement, exercise, anoxia, and different degrees of fasting, marked fluctuations in

[1] This is sufficient thiamine to cure opisthotonus, prevent the development of symptoms of chronic deficiency, and to lead to slow repair of those birds which had previously developed leg weakness.

blood pyruvic and lactic acid values may occur. Nevertheless, a strict relation between the two is maintained, so that a normal relation can be expressed graphically or by formula. This was found true in humans, rats, and pigeons.

Thus although the actual level of pyruvate or lactate individually can serve as a measure of the above factors, and under carefully controlled conditions even reflect true changes in the metabolism of either component, only a deviation in the normal relation between the two components is rigorous proof of a more fundamental disturbance. Therefore the use of this relation eliminates the otherwise difficult decision as to whether a given pyruvate increase is due to a genuine disturbance of pyruvate metabolism or to changes in difficultly controlled experimental conditions.

Such a change in the blood lactate-pyruvate relations has been noted in pigeons during the course of acute and chronic thiamine deficiency, indicating a marked decrease in pyruvate breakdown. The use of this relation has in fact made it possible to note a disturbance of pyruvate metabolism early in acute thiamine deficiency and to distinguish with assurance between relatively small degrees of chronic thiamine deficiency in pigeons.

Since the colorimetric determination of lactic acid is so simple, even as compared to the pyruvate estimation, it is suggested that both lactate and pyruvate levels be considered rather than pyruvate alone to determine fundamental changes in pyruvate metabolism.

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A QUANTITATIVE METHOD FOR ETHYL ALCOHOL NORMALLY PRESENT IN BLOOD

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The present method was devised for the quantitative determination of ethyl alcohol normally present in blood. It was our intention that the method conform to the following requirements. (*a*) Not more than 5 ml. of blood sample should be required for each determination. (*b*) No preliminary precipitation or distillation of the blood should be necessary. (*c*) The method must be suitable for quantitative determination of as little as 0.05 mg. of alcohol in 5.0 ml. of blood. (*d*) The chemical reaction involved in the method must be specific for the ethoxy group.

All methods based upon the oxidation of the alcohol by dichromate or permanganate had to be rejected in dealing with an alcohol content as low as 0.05 to 1.0 mg., because blood contains traces of volatile organic substances other than ethyl alcohol which also reduce the oxidant and therefore abnormally high values are always obtained.

The only method described in the literature which seemed to be adaptable for the determination of such small quantities of alcohol was Pregl's adaptation of the Zeisel reaction for the alkoxy group, as applied to tissues and blood by Gettler, Niederl, and Pichler (1) (hereafter designated as the G.-N.-P. method). Their procedure suggested the possibility of quantitatively determining the alcohol by introducing the blood sample directly into the side arm test-tube (*C*) of their apparatus ((1) Fig. 8, p. 192), thereby eliminating preliminary steam distillation. A study of the method, when applied to blood directly, indicated that several changes in apparatus and procedure were necessary.

The difficulties encountered in using the G.-N.-P. technique on blood directly were as follows: (1) When attempts were made to distil the alcohol from the whole blood contained in the side arm test-tube (*C*) of the G.-N.-P. apparatus with a stream of carbon dioxide, much frothing ensued, and as a result the blood was mechanically carried over into the hydriodic acid bulb (*E*) within a few minutes. The addition of chemical reagents did not eliminate the frothing. This made it necessary to devise some mechanical means to prevent the frothing. (2) The gravimetric determination of the alcohol as silver iodide was not only time-consuming, but the appreciable solubility of the silver iodide introduced quite an error with the small

quantities of alcohol that the method was intended for. It was found advantageous to supplant the gravimetric procedure with the titration method of Vieböck and Brecher (2). (3) The washing solution composed of sodium thiosulfate and cadmium sulfate was not satisfactory. Since a longer time was required to distil the alcohol from whole blood, a considerable quantity of hydriodic acid was carried over into the washing solution, decomposing the thiosulfate, with the production of colloidal sulfur and the precipitation of cadmium sulfide. When this occurred, it was found that the results obtained deviated considerably from the theoretical values. It was therefore necessary to substitute a washing solution in which this decomposition did not occur. (4) The wash chamber (G) was not large enough to hold all the water which collected during the distillation of the 5 ml. blood sample. When the wash chamber became filled with the aqueous distillate, there always existed the potential danger that some of the wash solution would be carried over into the receiving tube (I). (5) The space between the side arm (D) of the test-tube and the side tube of the bulb (E) containing the hydriodic acid presented a dead space in which some ethyl iodide vapors became trapped. (6) The bore of the inlet tube to the wash chamber (G) was too narrow. Iodine vapors from the hydriodic acid sometimes condensed and clogged up the tube. (7) The quantitative removal of any adhering reaction product from the end of the delivery tube (H) was very troublesome.

The difficulties described above, which were encountered with the G.-N.-P. technique when applied to blood directly, were eliminated in the following method.

Method in Detail

Description—The method is based on the conversion of the ethyl alcohol into ethyl iodide by interaction with hydriodic acid. The alcohol is distilled directly from the blood sample into hot hydriodic acid solution by means of a stream of carbon dioxide. The following reaction takes place, $C_2H_5OH + HI \rightarrow C_2H_5I + H_2O$. The ethyl iodide formed passes through a washing chamber containing a suspension of lead thiosulfate and basic lead carbonate. These reagents remove all iodine, hydrogen iodide, and hydrogen sulfide, which may distil along with the ethyl iodide. The ethyl iodide vapors pass through the wash chamber unaffected and are absorbed in a solution of potassium acetate in glacial acetic acid to which a little liquid bromine has been added. The ethyl iodide reacts with the bromine in the absorbing solution to form ethyl bromide and iodine bromide, $C_2H_5I + Br_2 \rightarrow C_2H_5Br + IBr$. When the distillation is completed, the IBr in the receiving tube is titrated by a modification of the procedure described by Vieböck and Brecher (2) for methyl iodide.

The titration is carried out after water is added to the absorbing solution containing the iodine bromide. In the presence of water, the excess bromine oxidizes the iodine bromide to iodate, $IBr + 3H_2O + 2Br_2 \rightarrow HIO_3 + 5HBr$. Any excess bromine remaining in the solution is removed by boiling. When cool again, potassium iodide is added, the following reaction taking place, $2HIO_3 + 10KI + 10HAc \rightarrow 10KAc + 6H_2O + 6I_2$. 6 atoms of iodine are liberated for each molecule of iodate, and hence for each molecule of ethyl iodide, thereby increasing the sensitivity of the reaction 6-fold. The liberated iodine is titrated with standard sodium thiosulfate solution. The ethyl alcohol is calculated from the quantity of standard thiosulfate used in the titration.

Reagents—

Hydriodic acid (sp. gr. 1.70, specially prepared for alkoxy determinations).

Tartaric acid (powdered).

Sodium sulfate (anhydrous, powdered).

Potassium hypophosphite (KH_2PO_2) (crystals or powder).

Potassium iodide (crystals).

Sulfuric acid (concentrated).

Starch solution (freshly prepared, 2 per cent).

Bromine (liquid).

Potassium acetate solution (25 per cent); dissolve 166.6 gm. in 500 ml. of distilled H_2O.

Potassium acetate (10 per cent) in glacial acetic acid; dissolve 55.5 gm. in 500 ml. of glacial acetic acid.

Sodium carbonate solution (10 per cent).

Lead thiosulfate-basic lead carbonate suspension. Add 0.2 gm. of lead thiosulfate and 0.8 gm. of basic lead carbonate (finely powdered) to 50 ml. of distilled water, contained in a 60 ml. dropping bottle, which has a medicine dropper graduated to deliver 2 ml. Shake thoroughly each time before pipetting the suspension. The latter is an improvement over the sodium thiosulfate-cadmium sulfate wash solution in that the hydriodic acid which passes into the wash chamber is immediately neutralized by the lead carbonate, thus preventing the decomposition of the thiosulfate and any further reaction with the cadmium sulfate. In addition, both the iodine and hydriodic acid are removed from the solution by precipitation as insoluble lead iodide. The low concentration of dissolved salts in the wash solution eliminates the possible decomposition of the ethyl iodide which may occur in high salt concentrations at elevated temperatures.

Sodium thiosulfate, approximately 0.01 N solution (to be standardized). Dissolve 2.48 gm. of $Na_2S_2O_3 \cdot 5H_2O$ in boiled, then cooled, distilled water, add 1 ml. of approximately 0.1 N sodium hydroxide, and dilute to 1 liter.

Iodate (standard solution). Accurately weigh 0.150 gm. of pure and dry $KH(IO_3)_2$. Transfer the weighed material, quantitatively, into a 500 ml. volumetric flask with the aid of distilled water. When the iodate has dissolved, add water to the mark and mix the contents. (10.0 ml. = 3.0 mg. of $KH(IO_3)_2$.)

Standardization of 0.01 N Thiosulfate Solution by Means of Standard Iodate Solution—Pipette 10 ml. of the standard iodate solution into a 125 ml. Erlenmeyer flask. Add 5 ml. of 25 per cent potassium acetate solution, 70 ml. of distilled water, 2 drops of concentrated sulfuric acid, and 1.5 gm. of solid potassium iodide. Allow to stand 15 minutes, and titrate with the thiosulfate solution (to be standardized) until the color is light yellow. Add 1.0 ml. of starch solution and continue the titration dropwise to the colorless end-point. The alcohol equivalent (in mg.) per ml. of thiosulfate solution = $(0.236 \times 3.0)/$(ml. thiosulfate used).
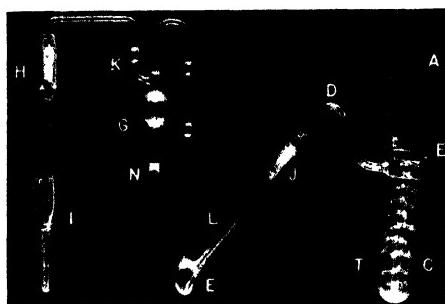
FIG. 1. The ethoxy apparatus

Ethoxy Apparatus[1] —The apparatus as designed for the direct determination of alcohol in blood is shown in Fig. 1. The changes introduced in the G.-N.-P. apparatus are as follows: The capacity of the wash chamber (G) was increased to 10 ml.; this permitted the blood sample in tube C to be distilled even to complete dryness. A bulb (K) was introduced to prevent any of the wash solution from being carried into the absorbing solution in I. The delivery tube (H) was made removable by introducing a ground glass joint; this facilitates the quantitative removal of the reaction product adhering to the tube. The bore of the delivery tube (N) leading into the wash chamber (G) was made large enough (1.5 mm.) to prevent any sublimed iodine from clogging up the tube. The bore of the side tube (L) leading into bulb E was reduced to 1 mm., and the upper end of the tube was supplied with a ground glass joint (J) to connect with the side arm of the test-tube (D). This eliminated the dead space previously

[1] Made by Eck and Krebs, New York; catalogue No. 3000A.

mentioned. A glass tree tube (T) was introduced to subdue the frothing. The glass tree consisted of small glass spurs fused onto a 5 mm. bore glass tube. As the froth rises in tube C, it is broken up by the glass spurs.

A small mineral oil bath was substituted for the micro burner used to heat the hydriodic acid in bulb E. The burner caused considerable bumping and therefore required constant attention. The Kipp generator (not shown in the picture) was replaced by a tank of carbon dioxide, because it was not possible to get sufficient pressure from the generator. A carbon dioxide bubble counter (not shown) consisting of a wash bottle containing 10 per cent sodium carbonate solution was introduced between the carbon dioxide tank and the ethoxy apparatus, the purpose being to indicate the rate of carbon dioxide flow, and also to remove any possible acid impurities. A constant leveling device was attached to the boiling water bath (not shown) in which the test-tube (C) was submerged.

Method of Charging and Assembling Apparatus—The carbon dioxide bubble counter, water bath with constant leveling apparatus, and mineral oil bath are mounted on a stationary stand in such a manner that the bulb E and tube C fit into the mineral oil and water baths respectively. When the water bath is at boiling temperature and the mineral oil bath between 130–135°, the ethoxy apparatus is charged and assembled as follows: Remove the stopper at the bottom of the wash chamber (G), hold the latter in a horizontal position, and introduce 2 ml. of the previously well shaken lead thiosulfate-basic lead carbonate suspension; then replace the stopper. Connect the delivery tube (H) to the tube leading from the wash chamber (G) by means of the ground glass joint. Introduce 1.5 ml. of the hydriodic acid solution through the side tube (L) into bulb E. Add a crystal of potassium hypophosphite, if the hydriodic acid has a deep brown color, due to the presence of an excessive amount of free iodine. In the receiving tube (I) place 3 ml. of the potassium acetate in glacial acetic solution and 0.1 ml. of liquid bromine. Pipette 5 ml. of the sample (oxalated blood) (if from an alcoholic use 1.0 ml. of blood and 4.0 ml. of water) into the side arm tube (C) and add 0.1 gm. of powdered tartaric acid. Shake the tube with a rotating motion until the blood turns dark brown and becomes quite viscous. The addition of saponin is not necessary. Add 3 gm. of anhydrous sodium sulfate, and mix with the glass tree tube (T), taking care not to get any of the blood on the upper walls of the test-tube. When the contents are well mixed, the glass tree tube is put in position by means of the rubber stopper, with the bottom of the tree tube extending to about ⅛ inch from the bottom of the test-tube. The side arm (D) of the test-tube is then connected to the side tube (L) by means of the ground glass joint (J). The top of the glass tree tube (A), by means of rubber tubing, is connected to the carbon dioxide bubble counter, which in turn is connected

with the carbon dioxide tank. The carbon dioxide pressure is so regulated that about one or two bubbles per second pass through the bubble counter. The delivery tube (*H*) is quickly lowered into the solution contained in the receiving tube (*I*). The hydriodic acid in bulb *E* is allowed to attain the temperature of the oil bath (130–135°) in which it is submerged. The side arm test-tube *C* is slowly lowered into the boiling water bath. The apparatus is then clamped into position with the side arm tube *C* submerged in the boiling water bath, and bulb *E* suspended in the mineral oil bath about 1½ inches below the surface of the oil. The carbon dioxide pressure is regulated while the blood is coagulating, and then set so that about two bubbles per second pass through the wash chamber (*G*). The apparatus requires no further attention until approximately 1½ hours later. Experiments have shown that during this interval the alcohol in the blood sample has been distilled in the form of ethyl iodide into the receiver tube (*I*). The delivery tube (*H*) and receiver tube (*I*) are then removed by disconnecting the ground glass joint (*H*).

Titration of Ethyl Iodide Formed—The contents of the receiving tube (*I*) are transferred into a 125 ml. Erlenmeyer flask. The delivery tube (*H*) and receiving tube (*I*) are washed with 5 ml. of the potassium acetate solution and then with 25 ml. of water. The washings are added to the main solution. The excess bromine is removed by boiling the contents of the flask over a free flame with a rotating motion. When the solution is water-clear, the flask and contents are cooled. 50 ml. of water and 1.5 gm. of solid potassium iodide are added and allowed to stand 15 minutes before titration of the liberated iodine. The 0.01 N thiosulfate solution contained in a burette is gradually added until the color of the solution becomes a faint yellow. 1 ml. of starch solution is then added and the titration continued until colorless.

The method has been applied to blood and urine samples from alcoholic persons with very good results. In such cases only 1 ml. of blood is to be used for each determination, and 0.1 N sodium thiosulfate should be used for the titrations. Before the alcohol content is calculated from the titration figure, a blank must be subtracted. This blank is obtained by using distilled water instead of blood and proceeding exactly as in the main analysis. The titration blank obtained should always be less than 0.2 ml. when 0.01 N thiosulfate solution is employed. Blank determinations are only necessary when a new set of reagents is used. The quantity of ethyl alcohol that is present in the 5 ml. blood sample is obtained by multiplying the ml. of 0.01 N thiosulfate solution used in the titration by its alcohol equivalence (mg. of C_2H_5OH per ml.), as previously indicated.

In Table I are presented some of the results obtained by the method described when applied to extremely dilute alcohol solutions in pure water.

4 gm. of anhydrous C_2H_5OH were accurately weighed in a weighing bottle and then dissolved in water and made up to a volume of 1000 ml. (Solution A). After thorough mixing, 10 ml. of Solution A were transferred to a 1000 ml. volumetric flask and water was added to the mark and thoroughly mixed (Solution B). In the series of analyses reported in Table I the following ml. of Solution B were used respectively: 5.00, 5.00, 3.75, 2.50, 1.25, 1.00, 0.63. In each case, if necessary, water was added to bring the total volume up to 5 ml. This series of solutions, having an alcohol content as indicated in the second column of Table I, was then analyzed by the method described above. The results, charted in Table I, indicate that the analyti-

TABLE I

Results Obtained on Solutions of Alcohol in Pure Water

Sample No.	C_2H_5OH present in 5.0 ml. H_2O	C_2H_5OH found by analysis	Average	Error in C_2H_5OH
	mg.	*mg.*	*mg.*	*mg.*
1-a	0.200	0.202	0.195	−0.005
1-b	0.200	0.188		
2-a	0.200	0.212	0.200	0.000
2-b	0.200	0.189		
3-a	0.150	0.142	0.150	0.000
3-b	0.150	0 158		
4-a	0.100	0.093	0.102	+0.002
4-b	0.100	0.110		
5-a	0.050	0.053	0.054	+0.004
5-b	0.050	0.056		
6-a	0.040	0.047	0.043	+0.003
6-b	0.040	0.039		
7-a	0.025	0.030	0.026	+0.001
7-b	0.025	0.023		

cal findings agree quite closely with the actual quantities of alcohol present (deviation ±0.005 mg.).

In Table II are recorded some of the results obtained by the method when applied to blood to which definite quantities of alcohol were added. For each set about 50 ml. of normal blood were taken. Two 5.0 ml. samples of the blood were analyzed before the addition of alcohol. To the remaining blood (known volume) definite quantities of C_2H_5OH were added by means of a micro weighing pipette. From these data the quantity of added alcohol in 5.0 ml. of blood was calculated.

From the data put forth in Table II it becomes evident that there is a small loss of alcohol. This loss, however, is somewhat proportional to the quantity of alcohol present in the sample. The average loss is 12.5 per cent. Considering that we are dealing with very small quantities of

alcohol (1 mg. and less), a loss of this order is not as important as it seems. It is advisable to make a correction for this loss by multiplying the quantity of alcohol actually found by 1.14.

Direct Standardization of Thiosulfate Solution against Known Quantities of Alcohol Added to Blood—In order to avoid the introduction of correction factors (indicated above) in the calculation of the quantity of alcohol present, direct standardization of the thiosulfate solution against known quantities of alcohol added to blood samples may be resorted to.

TABLE II

Results Obtained on Samples of Blood to Which Were Added Definite Quantities of Alcohol

Sample No.	C₂H₅OH added to 5.0 ml. blood	C₂H₅OH found by analysis	Original alcohol content, average	C₂H₅OH found minus original alcohol content	Average	Error
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1-a	0.000	0.055	0.063			
1-b	0.000	0.070				
1-c	0.078	0.125		0.062		
1-d	0.078	0.128		0.065		
1-e	0.078	0.131		0.068	0.065	−0.013
2-a	0.000	0.115	0.100			
2-b	0.000	0.085				
2-c	0.169	0.250		0.150		
2-d	0.169	0.256		0.156	0.153	−0.016
3-a	0.000	0.170	0.170			
3-b	0.483	0.568		0.398		
3-c	0.483	0.591		0.421		
3-d	0.483	0.611		0.441	0.420	−0.063
4-a	0.000	0.056	0.058			
4-b	0.000	0.060				
4-c	0.890	0.828		0.770		
4-d	0.890	0.839		0.781		
4-e	0.890	0.844		0.786	0.779	−0.111

30 ml. of blood from non-alcoholics (a composite well mixed sample from several persons may be used) are collected. Duplicate determinations on two 5 ml. portions are made for the normal alcohol content by the method described above. A series of such determinations indicated that the normal alcohol in 5.0 ml. of blood requires from 0.80 to 1.50 ml. (average 1.15 ml.) of the 0.01 N thiosulfate solution. By means of a micro weighing pipette an accurately weighed amount (order of 5.0 mg.) of ethyl alcohol was added to the remaining 20.0 ml. of the blood sample. From these data the quantity of ethyl alcohol that was added to each 5.0 ml. of

blood sample was calculated. Triplicate determinations on 5.0 ml. samples of this blood were now made for alcohol by the method described. For the titration of the liberated iodine the 0.01 N thiosulfate solution was used. The average value of three determinations was used in calculating the C_2H_5OH equivalent (in mg.) per ml. of 0.01 N thiosulfate solution. The 1.15 ml. of 0.01 N thiosulfate solution needed for the normal alcohol that is present in the blood was subtracted from the ml. of thiosulfate used in the titration of the blood to which a definite quantity of alcohol was added. On the above basis, it was found that 1.0 ml. of 0.01 N thiosulfate solution equals 0.086 mg. of C_2H_5OH. In order to obtain the mg. of alcohol in 5 ml. of blood, this equivalent 0.086 is multiplied by the ml. of 0.01 N thiosulfate used in the titration.

SUMMARY

A quantitative method for ethyl alcohol in blood, based upon the alkoxy reaction, is presented.

The outstanding features of the method are as follows:

1. Only 5.0 ml. of blood are required for the determination.

2. No preliminary precipitation or distillation of the blood is necessary.

3. Quantities of alcohol as small as 0.02 mg. can be quantitatively determined.

4. The method is specific for the alcohol (OH) group.

5. The method may be used for the analysis of blood and urine from alcoholic individuals.

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A POLAROGRAPHIC CHARACTERIZATION OF NICOTINIC ACID AND RELATED COMPOUNDS

I. PYRIDINE AND NICOTINIC ACID*

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The tertiary nitrogen of pyridine and nicotinic acid is methylated to the corresponding quaternary ammonium derivative when these compounds are fed to animals (1, 2). Quantitative studies of this reaction have not been satisfactory owing to the fact there are no adequate methods for the analysis of the methylated derivatives in the urine. Preliminary experiments showed that both methylpyridinium ion and trigonelline are reducible at the dropping mercury cathode and may be analyzed in pure solution by the polarographic technique. Before attempting to make use of this fact for the development of a method of determining the methylated compounds in urine, we considered it advisable to make a general survey of the polarographic behavior of several members of the pyridine series.

The present paper deals with the electrolysis of pyridine and nicotinic acid in both buffered and unbuffered solutions. Some studies on the reduction of these compounds at the dropping electrode have already been reported (3–5).

Electrical Apparatus and Equipment—The electrical apparatus consisted of the Heyrovský-Shikata type of micro polarograph distributed by E. H. Sargent and Company. The current voltage curves were recorded on sensitized bromide paper in the usual manner. The voltage was applied at the rate of 0.187 volt per cm. and the current was measured by means of a galvanometer with a sensitivity of 4.95×10^{-8} ampere per cm. per 0.33 meter. All potentials were referred to the saturated calomel electrode by means of the cell designed by Lingane and Laitinen (6).

The dropping electrodes were made from a piece of marine barometer tubing. The $m^{\frac{1}{2}}t^{\frac{1}{6}}$ values, where m = the weight of mercury flowing per second and t = the drop time, were determined by direct measurement. The accuracy of the calibration was checked by electrolyzing a 0.001 M and a 0.003 M ZnCl₂ solution in 0.1 N KCl plus enough HCl to hold the Zn⁺⁺ in solution. The resulting diffusion current was then compared with that calculated from the Ilković equation. The experi-

mental values were 5.3 per cent and 5.1 per cent higher than the calculated value but in good agreement with the $+5$ per cent deviation given by Kolthoff and Lingane (7). Oxygen was removed by passing purified hydrogen gas through the solution for 20 minutes prior to electrolysis.

Preparation of Materials—Baker and Adamson c.p. pyridine (b.p. 113–115°) was stored 2 days over solid potassium hydroxide. It was then fractionated in an all-glass fractionating still. The liquid boiling between 114.5–115° was used to prepare the standard solutions. A 0.025 M solution was prepared by dissolving 1.980 gm. of pyridine in 30 ml. of 1 N HCl and diluting to 1 liter. 1 ml. is equivalent to 0.001 M pyridine in the solution used for analysis. Another standard was prepared by diluting 2 ml. of pyridine to 100 ml. This solution was used immediately after preparation. 1 ml. is equivalent to a 0.01 M pyridine solution in the solution used for the analysis (25 ml.).

Eastman's nicotinic acid was recrystallized three times from hot water and dried to constant weight at 110°. It was stored in the vacuum desiccator (m.p. 228.5–229°). A standard solution (0.025 M) was prepared by dissolving 0.3076 gm. of the purified nicotinic acid in redistilled water and diluting to 100 ml. The solution was preserved with toluene.

All solutions used in these studies were prepared with redistilled water. Potassium chloride and tetramethylammonium bromide were recrystallized twice from redistilled water before use. With these exceptions, commercial c.p. grade chemicals were used without further purification. Sodium and potassium phosphate buffers were made 0.6 M with respect to total phosphate content and were diluted as needed.

Procedure—The dilutions were prepared by pipetting the correct volume of the standard solution into a 25 ml. volumetric flask. 5 ml. of the salt solution, buffer, or HCl were then added and the flask filled to volume with redistilled water. These solutions were then electrolyzed after the removal of oxygen. The pH was measured with the glass electrode.

The residual current was measured by extending its slope except in those cases in which the current rise of the supporting electrolyte began at a potential more positive than the voltage at which i_d, the diffusion current, was measured.

The reported values of the diffusion current have been divided by the $m^{\frac{1}{2}}t^{\frac{1}{6}}$ value at the voltage selected for the measurement, thus making all of the data comparable.

Comparison curves of all of the supporting electrolyte solutions were made. The polarogram was considered to represent complete reduction of the reducible material when the sensitivity of the galvanometer was so adjusted that the final current rise of the test solution corresponded to that of the electrolyte alone.

Pyridine—Owing to the relatively high concentrations of pyridine that were used, the usual procedures employed in bubbling hydrogen through a solution containing a volatile compound were not considered satisfactory. Therefore, the supporting electrolyte was freed from air in a separatory funnel, and the solution was run into a volumetric flask containing the pyridine while the gas was still running. The resulting solution was immediately transferred to the electrolysis cell and the polarogram obtained. Such a solution must obviously contain a small amount of dissolved oxygen.

Electrolysis of Pyridine in Buffered Solutions—Practically no difference was noted between polarograms of the buffers alone and the test solutions when 0.005 or 0.001 M pyridine solutions were electrolyzed below pH 6. The solutions that were tested are given in Table I. A rather poorly defined wave was obtained when 0.1 M potassium phosphate buffers of pH 6.1 and 7.4 were used for the electrolysis. The current was proportional to the concentration of pyridine within about 10 per cent between the limits of 0.001 and 0.01 M pyridine. The half wave potential was -1.7 volts to the saturated calomel electrode. No maximum was observed.

A maximum was obtained when 0.005 M pyridine was electrolyzed in a 0.1 M sodium borate buffer of pH 8.7. No attempt was made to suppress it. The half wave potential of pyridine in this solution was about -1.8 volts.

No difference between the polarogram of the test solutions and the supporting electrolyte was observed when 0.005 M pyridine was electrolyzed in a 0.1 M potassium phosphate buffer of pH 12 or in 0.1 N sodium hydroxide solution. Polarograms of pyridine in several alkaline buffers are shown in Fig. 1.

Electrolysis of Pyridine in Unbuffered Salt Solutions—The observations of Shikata and Tachi (4) regarding the behavior of a solution that was 0.1 M with respect to KCl, 0.001 M with respect to HCl, and 0.01 M with respect to pyridine have been confirmed. The electrolysis of a solution of 0.001 M HCl in 0.1 M KCl (pH 2.9) gave a diffusion wave of 16.9 microamperes with a half wave potential of -1.49 volts to the saturated calomel electrode. When a similar solution but containing 0.01 M pyridine was used (pH 5.6), this wave was reduced to 6.48 microamperes but neither its half wave potential nor its slope was changed. When less hydrochloric acid was used, so that the pH was 6.2, two waves were found. The first was 1.09 microamperes at -1.51 volts, and the second was 5.28 microamperes at -1.78 volts $(E_{\frac{1}{2}})$.[1]

Good waves were obtained when pyridine was electrolyzed in 0.1 M KCl solution in the absence of added acid or alkali (pH 7 to 8). The

[1] $E_{\frac{1}{2}}$ is the half wave potential.

current was proportional to the concentration of pyridine within 10 per cent.

TABLE I

Values Obtained for i_d and $E_{\frac{1}{2}}$ of 0.001 M Pyridine and of Nicotinic Acid in Different Solutions

Solution, buffered	Pyridine			Nicotinic acid	
	pH	i_d	$E_{\frac{1}{2}}$	i_d	$E_{\frac{1}{2}}$
		microamperes		*microamperes*	
0.4 N HCl.......		No wave		5.90	−1.1
0.1 "　"	1	"　　"		5.10	−1.1
0.2 M K citrate-phosphate	3.3	"　　"		*	0.9–1.2
0.1 "　" phthalate　.........	3.3	"　　"		†	
0.1 "　" phosphate	6.1	11.85	−1.69	†	
0.3 "　"　"　... ...	7.0	†		2.82*	−1.55
0.1 "　"　"　.　....	7.4	11.28	−1.69	†	
0.1 " tetramethylammonium phosphate .　........... .	7.0	17.08	−1.7	†	
0.3 M K phosphate.　......	8.0	†		2.57*	−1.57
0.6 " Na borate　..　.......	8.7	†		0.98	−1.66
0.1 "　"　"　..........	8.6	12.4	−1.8	0.65	−1.63
0.3 " K phosphate .　.......	9.1	†		0.77*	−1 68
0.3 "　"　"　....... ..	10	†		No wave	
0.3 "　"　"　...,	12	No wave‡		"　　"	
0.1 N NaOH　..... .	13	"　"　‡		"　　"	
0.1 " KCl　.....　........	3.2	†		7.0	Double wave
0.1 "　"　..	7.0	0.98	−1.8	0.4	−1.1, −1.3, −1.5
0.1 "　"　.....　..	9.2	†		No wave	
0.1 " tetramethylammonium bromide .　... .　.... .	7.0	1.12	−1.8	†	
0.3 M Na acetate　..	7.0			1.65	−1.63
0.1 "　"　"　.　.... .	6.6			1.32	−1.52
0.5 " NaHCO₃　.　... ..	8.1			1.55	−1.62

The i_d values have been divided by $m^{\frac{2}{3}}t^{\frac{1}{6}}$. The $E_{\frac{1}{2}}$ values are with reference to the saturated calomel electrode.

* Catalytic reduction of H^+ interferes.

† Compound not tested in this solution.

‡ Some reduction was observed when 0.05 M pyridine was electrolyzed in these solutions.

The half wave potential of pyridine in this solution was −1.8 volts. It was found to be independent of the concentration of pyridine. Qualitatively, $E_{\frac{1}{2}}$ shifted to more negative potentials with increasing pH. This shows that H^+ takes part in the electrode reaction. The diffusion currents

and half wave potentials of 0.001 M pyridine in various buffered and un-buffered solutions are also shown in Table I.

The use of the polarograph cannot be recommended for the analysis of pyridine. However, if it should be found convenient to determine pyridine polarographically, heavily buffered solutions (*i.e.*, at least 0.1 N) should be used. Sodium or potassium phosphate buffers in the pH range 6 to 8 have proved most suitable.

Electrolysis of Nicotinic Acid in Buffered Solutions—The electrolysis of nicotinic acid in potassium phosphate buffers more acid than pH 10 gave rise to a reduction wave that did not develop into a diffusion wave. Dis-charge of hydrogen from the buffer interfered. Comparison of the test polarograms with those of the buffer showed that this deposition occurred
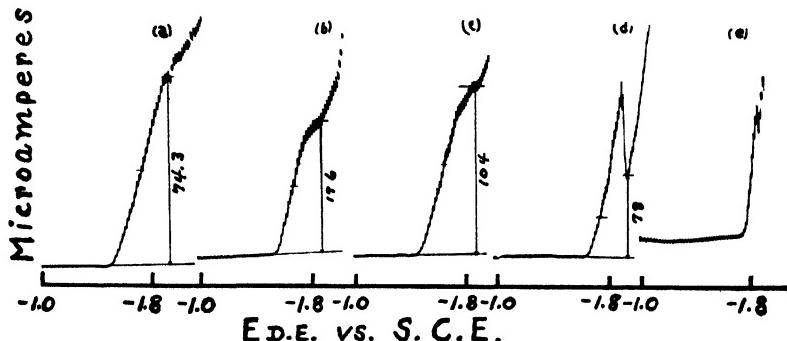
Fig. 1. Polarograms of pyridine in neutral and alkaline buffers. (*a*) 0.005 M pyridine in 0.1 M sodium phosphate buffer, pH 6.1; (*b*) 0.001 M pyridine in 0.1 M sodium phosphate buffer, pH 7.5; (*c*) 0.005 M pyridine in 0.2 M tetramethylammonium phosphate buffer, pH 7.0; (*d*) 0.005 M pyridine in 0.1 M sodium borate buffer, pH 8.7; (*e*) 0.005 M pyridine in 0.1 M sodium phosphate buffer, pH 12. $E_{D.E.}$ = potential, in volts, of the dropping electrode; $S.C.E.$ = saturated calomel electrode.

at a potential about 0.2 to 0.3 volt more positive than the deposition of hydrogen from the buffer alone. This indicates that the products of re-duction in the phosphate solutions catalyzed the deposition of hydrogen from the buffer. This effect was not investigated further, but it should be pointed out that such catalytic reactions often allow the determination of the catalyst in concentrations as low as 10^{-7} to 10^{-8} M.

No reduction was observed when nicotinic acid was electrolyzed in solutions as alkaline as pH 10 or higher (Table I). The best waves of nicotinic acid in buffered solutions were obtained when sodium borate buffer between pH 8 and 9 was used as the supporting electrolyte. The same observation was made by Lingane and Davis (5). The current was not proportional to the concentration of nicotinic acid unless the buffer was at least 0.6 M.

The half wave potential of nicotinic acid was found to be dependent on both the pH and the buffer. In the phosphate solutions the $\Delta E/\Delta pH$ shift was about 90 millivolts per pH unit.

Electrolysis of Nicotinic Acid in Unbuffered Salt Solutions—The polarograms of nicotinic acid in 0.1 M potassium chloride solutions showed double waves in acid solutions (pH 2.2). The height of the wave decreased sharply as the pH was increased and disappeared when the nicotinic acid was all in the form of the potassium salt, pH 9.2 (Table I).

Very nicely formed waves were obtained when nicotinic acid was added to solutions of the sodium salts of weak acids, such as acetic, citric, and carbonic acids. The ratio of current to concentration increased with increasing concentration of nicotinic acid, and was greater in 0.5 M salt
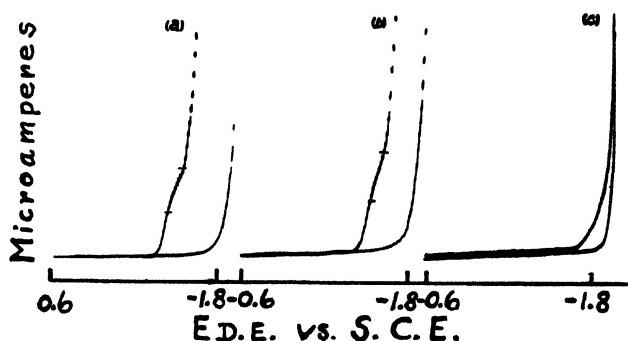
FIG. 2. Polarograms of nicotinic acid in 0.3 M potassium phosphate buffers. (a) 0.0049 M nicotinic acid, pH 7.0; (b) 0.002 M nicotinic acid, pH 8.0; (c) 0.002 M nicotinic acid, pH 10.0. $E_{D.E.}$ = potential, in volts, of the dropping electrode; *S.C.E.* = saturated calomel electrode.

solutions than it was in 0.1 M salt solutions. The half wave potentials became more positive with increasing concentration of the acid. These effects are to be correlated to the increasing acidity of the solutions. Only single waves were observed in the alkaline solutions.

Effect of Buffer Capacity and pH on i_d—The height of the nicotinic acid wave was found to be the resultant of two *independent* factors. These were (a) the concentration of nicotinic acid; (b) the pH at the surface of the electrode. The second factor is in turn influenced by two independent factors: (a) the pH of the solution; (b) the buffer capacity of the solution. Therefore, in practical work, the diffusion current will depend on all three factors. Proportionality of current to concentration of nicotinic acid is observed only when the pH at the electrode surface is held constant during the course of the electrolysis, and the magnitude of the current is greater the lower the pH.
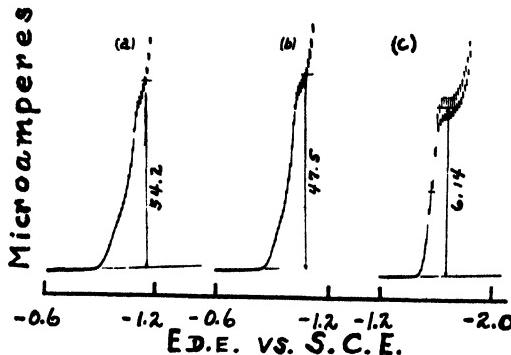
FIG. 3. Polarograms of nicotinic acid in HCl and in sodium borate buffer. (a) 0.0049 M nicotinic acid in 0.4 N HCl; (b) 0.0049 M nicotinic acid in 0.1 N HCl; (c) 0.005 M nicotinic acid in 0 6 M sodium borate buffer, pH 8.7. The residual current was measured in a separate experiment to determine the diffusion current in the HCl solutions. $E_{D,E.}$ = potential, in volts, of the dropping electrode; S.C.E. = saturated calomel electrode.

TABLE II

Relation of Current to Concentration of Nicotinic Acid

Concentration of nicotinic acid	0.4 M HCl		0.1 N HCl		0.6 M Na borate, pH 8.7	
	i_d*	i_d:C	i_d*	i_d:C	i_d	i_d:C
mM per l.$^{-1}$	micro-amperes	microamperes per mM^{-1} per l.$^{-1}$	micro-amperes	microamperes per mM^{-1} per l.$^{-1}$	micro-amperes	microamperes per mM^{-1} per l.$^{-1}$
0.1	0.578	5.78				
0.5	2.99	5.94	2.58	5.16		
1.0	5.90	5.90	5.10	5.10	0.98	0.98
3.0	17.80	5.90			3.46†	(1.15)
5.0	29.10	5.92	25.7	5.15	4.90	0 97
10.0	58.8	5.88			10.00	1.00
Average............		5.90 ± 0.08		5.13 ± 0.03		0.98 ± 0.05

The i_d values have been divided by $m^{\frac{1}{3}}t^{\frac{1}{6}}$.

* The empirical method must not be used since the final current rise of the acid begins at a slightly more positive potential than the voltage at which the diffusion wave can be measured. The residual current was measured separately.

† A single determination; other values are averages of triplicates.

Constant i_d:C ratios were observed in only three of the supporting electrolytes studied. These were 0.4 N HCl (K^2 = 5.90 microamperes), 0.1 N HCl (K = 5.10 microamperes), and 0.6 M sodium borate buffer of pH 8.7 (K = 0.98 microampere).

² K = the diffusion current constant = i_d/C where C = mM per liter.

The i_d of a 0.001 M solution of nicotinic acid in 0.1 M sodium borate buffer was 0.65 microampere and the ratio of $i_d:C$ increased with increasing concentration of nicotinic acid.

The wave height of a 0.001 M nicotinic acid solution progressively decreased as the pH was increased in the buffered solutions and finally disappeared at pH 10.

The wave height of 0.001 M nicotinic acid decreased in the following order in solutions of approximately the same pH: 0.3 M sodium acetate solution, 0.1 M sodium acetate solution, and 0.1 M potassium chloride solution (pH 7).

Polarograms of nicotinic acid are shown in Figs. 2 and 3.

The polarograph may be used for the determination of nicotinic acid. However, owing to the extraordinary effect of pH and buffer capacity on the diffusion current constant, extreme care must be used in its application. The concentration of H+ at the electrode surface must be maintained constant at all times during a determination. The best current-concentration values were obtained in acid solutions. Although good waves could be obtained in sodium acetate, citrate, and bicarbonate solutions, the precision was not sufficiently high to warrant analytical determinations. Data showing the relation of current to concentration of nicotnic acid are given in Table II.

DISCUSSION

Shikata and Tachi studied the reduction of pyridine at the dropping electrode in unbuffered salt solutions (3). They found two waves, the first at about −1.5 volts to the saturated calomel electrode and the second at about −1.7 volts, when the electrolysis was carried out in a solution that contained 0.1 M KCl, 0.001 M HCl, and 0.0125 M pyridine. They attributed the first wave to the reduction of the pyridinium ion and the second to the reduction of the undissociated molecule. These authors used the shift of the tangent potential to distinguish the pyridine waves from the supporting electrolyte current. Their polarograms did not show a diffusion wave at −1.7 volts.

These observations have been confirmed, but it is doubtful that the first wave represents the reduction of pyridinium ion instead of hydrogen ion. We have observed that when pyridine was added directly to a 0.1 M KCl solution, the half wave potential of the resulting wave was found to be −1.77 volts. The half wave potential of 0.001 M HCl in 0.1 M KCl was −1.49 volts. Adding pyridine to this solution did not change either the half wave potential or the slope of this hydrogen ion wave. This is the wave that Shikata and Tachi interpret as being due to the reduction of pyridinium ion.

If they are correct in their interpretation, buffering the solution should

either have no effect or increase the height of the first wave if the buffered and unbuffered solutions have the same pH. Instead, the wave at -1.5 volts was eliminated under these conditions, and only one wave at -1.7 volts was observed.

Electrode Reactions—Polarographic waves generally result from one of two causes: (*a*) direct reduction by a transfer of electrons from the electrode to the reducible material; (*b*) catalysis of the reduction of H^+ at the electrode, giving a "catalytic" wave. The height of the waves in the first case is controlled solely by diffusion of the reducible material. The only effect of buffering the solution or changing its pH is to affect the half wave potential if H^+ plays a rôle in the electrode reaction.

The height of the waves in the second case is dependent on both the concentration of H^+ at the electrode and the concentration of the catalyst. This height may be used to determine the concentration of one factor when the other factor is held constant.

In organic reductions, a third type of reaction is possible and many reactions are assumed to proceed by this path. This type involves reduction of H^+ at the electrode and then a secondary reduction of the reducible molecule by the nascent hydrogen. Thus, Shikata and Tachi postulated that pyridine was reduced to piperidine in unbuffered solutions according to the reactions

$$6H^+ + 6e \rightarrow 6H \text{ (electrode reaction)}$$
$$6H + C_5H_5N \rightarrow C_5H_{11}N \text{ (secondary reduction)}$$
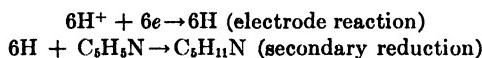
Kolthoff and Lingane question that 6 electrons actually take part in the electrode reaction under these conditions (8).

It has not been generally appreciated that if a diffusion wave is to be formed as a result of this mechanism the H^+ taking part in the reduction must be reduced at a more positive potential than the H^+ of the supporting electrolyte alone. This requires a catalytic mechanism in which the catalyst is not very active. The reduction of both pyridine and nicotinic acid appears to follow a mechanism of this sort.

The wave height will depend on the pH at the electrode surface as well as the concentration of reducible material and, under these conditions, the Ilković equation, $i_d = 605 \; nD^{\frac{1}{2}}Cm^{\frac{2}{3}}t^{\frac{1}{6}}$, where $n =$ the electron change, $D =$ the diffusion coefficient of the reducible material in the test solution, $C = $ the concentration of the reducible material in mM per liter, $m = $ the weight of mercury in mg. flowing through the capillary per second, and $t = $ the drop time, cannot be used to calculate the electron change.

The approximate diffusion coefficient of pyridine was calculated from the conductivity at infinite dilution (9) by means of the equation given by Kolthoff and Lingane (7, 8).

The *apparent* electron change for the reduction of pure pyridine in pure

potassium chloride (pH 7 to 8) was found to be 0.6, although the current was proportional to the concentration of pyridine. This substantiates the doubts of Kolthoff and Lingane. When the potassium chloride was replaced by 0.1 M potassium phosphate buffer of pH 6.1 or 7.4, the apparent electron change was found to be 6. The function of the buffer is obvious.

This effect was even more pronounced with nicotinic acid. Since no waves were observed when nicotinic acid was electrolyzed at pH 10 or higher, one concludes that the undissociated molecule is the catalyst for the primary reduction of H^+. The true catalytic nature of the reduction is apparent from the fact that the i_d of a 0.001 M solution of nicotinic acid is about 6 times greater in 0.4 M HCl than it is in 0.6 M sodium borate buffer of pH 8.7. The influence of the buffer capacity on the wave is seen in the lack of proportionality between the current and concentration of nicotinic acid in all solutions that were not heavily buffered. In the presence of phosphate ions, the first "reduction" wave was due to this type of reaction, but the products of the reduction strongly catalyzed the deposition of hydrogen from the buffer, and a true catalytic reduction of H^+ developed. One would expect that the half wave potentials of such reactions would be dependent on both the pH and the buffer, and that $E_{\frac{1}{2}}$ should be more positive in a buffered solution than in an unbuffered solution of the same pH. This was found to be true.

These observations enable us to explain an apparent discrepancy between the present results and those of Lingane and Davis (5). These authors found a wave with $E_{\frac{1}{2}}$ of -1.7 volts to the saturated calomel electrode when sodium nicotinate was electrolyzed in a 0.1 N KCl solution. We found no wave. We have found that the reaction in the alkaline region is very sensitive to the presence of buffers. A small amount of extraneous material in the solutions used by these authors could easily have caused a wave to appear. Since $E_{\frac{1}{2}}$ shifts rapidly with pH, the half wave potentials of nicotinic acid in any but heavily buffered solutions have no significance.

SUMMARY

The polarographic behavior of pyridine and nicotinic acid in both buffered and unbuffered solutions has been studied. It has been concluded that the mechanism of the electrode reaction involves the primary reduction of H^+ to H according to the reaction $nH^+ + ne \rightarrow nH$ (electrode reaction). The molecules are then reduced by the hydrogen that is formed, $R + nH \rightarrow RH_n$. The Ilković equation cannot be safely used to calculate the electron change.

It is concluded that pyridine is probably reduced to piperidine in unbuffered media. The contention of Shikata and Tachi that the pyridinium

ion is reduced at a more positive potential than the undissociated molecule could not be substantiated.

No information regarding the number of H^+ or electrons involved in the reduction of nicotinic acid was obtained. The i_d of nicotinic acid was found to be dependent on the pH and buffer capacity of the solution as well as on the concentration of nicotinic acid.

It is concluded that the nicotinic acid waves are due to the catalytic reduction of H^+ with the undissociated nicotinic acid molecule acting as a mild catalyst. The nicotinate ion was not found to be reducible.

Values for i_d and $E_{\frac{1}{4}}$ of both pyridine and nicotinic acid in several solutions have been reported.

The complete data are on file at the University of California library.

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GLUTAMINE AND GLUTAMIC ACID AS GROWTH FACTORS FOR LACTIC ACID BACTERIA

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In 1939 McIlwain, Fildes, Gladstone, and Knight found that the growth of *Streptococcus hemolyticus* depended upon the addition of glutamine to their medium, the presence of as little as 0.1 γ per ml. producing noticeable growth in 24 hours (1). *dl*-Glutamic acid also exhibited a growth action, but over 10,000 times as much was required for a similar effect. The suggestion was made that the organism might be able to synthesize glutamine from glutamic acid in high concentration, and that the essential growth activity dependent on glutamine was ammonia transfer, possibly similar to that found by Krebs (2) for animal tissues.

Fildes and Gladstone (3) noted that other organisms, in particular *Bacillus anthracis* and *Pneumococcus*, were also stimulated by glutamine and concluded that it probably has a general bearing upon bacterial growth. McIlwain (4) then reported that this effect was highly specific for glutamine, a number of related compounds and derivatives having no growth action for *Streptococcus hemolyticus*. Among these were several peptides of glutamine; whereupon he concluded that the organism probably lacked the enzymes for utilizing glutamine in protein synthesis, and therefore this mechanism could not be used to account for the growth action. He did not consider the ammonia transfer function very likely either, since the organism was unable to use glutamic acid except in high concentrations. Ammonium pyrrolidone α-carboxylate, the major breakdown product of glutamine in aqueous solution, was also without effect.

Preliminary observations in this laboratory had shown that glutamine had strong growth-promoting properties for certain lactic acid-producing bacteria on a synthetic medium, and we decided to investigate more thoroughly some of the relationships involved.

EXPERIMENTAL

The bacteria were grown in the liquid medium proposed by Pennington, Snell, and Williams (5). After 24 hours, the culture was centrifuged, the medium decanted, and the cells suspended in 0.9 per cent sterile saline solution. 1 drop of this cloudy suspension was added aseptically to each test culture, which contained 2.5 ml. of the medium and 2.5 ml. of aqueous glutamine or other addendum. Since glutamine is rapidly decomposed by

heating in aqueous solution (6), sterilization was always carried out by filtration through a Berkefeld filter. The desired amounts of glutamine were then added aseptically to the tubes, which had been previously autoclaved and cooled, and all were then inoculated and allowed to incubate at the appropriate temperatures for approximately 13 hours. The extent of growth was determined quantitatively by measuring the turbidities of the resulting cultures by means of the Williams thermoelectric turbidimeter (7).

1 liter of the medium contained the following substances: 10 mg. each of glycine, dl-alanine, l-leucine, dl-isoleucine, l-aspartic acid, dl-valine, l-histidine, l-tryptophane, l-proline, l-hydroxyproline, dl-serine, dl-threonine, l-tyrosine, l-cystine, dl-methionine, dl-β-phenylalanine, l-lysine, l-arginine,

TABLE I

Effect of Glutamine on Growth of Lactic Acid Bacteria

Gluta-mine	Amount of bacteria formed (wet cells per cc.)								
	S. lactis 125	S. lactis UT*	S. lactis 7963	S. lactis 374	S. lactis R	S. lactis R8043	L. pentosus 124.2	L. arabinosus 17.5	L. casei e
γ per cc.	γ	γ	γ	γ	γ	γ	γ	γ	γ
0.0	15	50	35	8	20	25	25	15	20
0.2	30	70	40	10	20	25	35	20	25
0.6	40	100	70	15	40	50	50	35	30
1.0	50	120	90	25	70	70	70	50	75
2.0	80	260	170	40	110	100	110	90	75
3.0	105	380	220	45	165	160	140	135	70
4.0	175	500	290	70	210	200	190	165	70
5.0	245	600	300	85	270	260	210	185	70
10.0	240	650	330	45	190	180	280	275	75

* This strain was isolated from milk at the University of Texas.

l-asparagine, adenine, guanine, uracil, thymine, and xanthine, 20 gm. of glucose, 12 gm. of sodium acetate, 1.0 gm. each of potassium mono- and dihydrogen phosphates, 0.4 gm. of magnesium sulfate heptahydrate, 0.02 gm. each of sodium chloride, ferrous sulfate heptahydrate, and manganous sulfate monohydrate, 5 mg. of inositol, 200 γ each of thiamine, pyridoxine, calcium pantothenate, riboflavin, and nicotinic acid, 0.4 γ of biotin, and 5.0 γ of folic acid concentrate (8). The pH of the medium was adjusted to 6.7 to 6.8 before use.

DISCUSSION

The striking growth effects produced by the addition of glutamine to the medium are shown in Table I. In every case, there was practically no

growth without glutamine, and a definite response to 0.2 to 0.6 γ per ml. in a 13 hour test. It is highly significant that this lower limit of effectiveness was the same for all the organisms tested, and is in essential agreement with the lower limit found by McIlwain et al. (1) for *Streptococcus hemolyticus*. From the constancy of this effect, it appears likely that the function is the same in all cases.

TABLE II

Effect of l(+)-Glutamic Acid on Growth of Lactic Acid Bacteria

Glutamic acid	Amount of bacteria formed (wet cells per cc.)								
	S. lactis 125	S. lactis UT	S. lactis 7963	S. lactis 374	S. lactis R	S. lactis R8043	L. pentosus 124.2	L. arabinosus 17.5	L. casei ε
γ per cc.	γ	γ	γ	γ	γ	γ	γ	γ	γ
0.0	15	50	35	8	20	25	25	15	20
0.2					20	35	30	15	30
0.6							30	30	
1.0	20	45			25	50	50	55	50
2.0	20	40			110	145	110	95	
3.0					210	225	160	150	75
4.0								180	
5.0	20	55			275	320	230	200	75
10	40	80	60		270	250	270	290	75
15	55	100	75				290		
20	80	215	90	30	240				70
30			90						
50	155	430	160						
60				35					
100	185	500	210	50					70
160				60					
200			270	70					70
300				75					
400			280						
500				110					
1000				130					

To gain more insight into the problem, the ability of glutamic acid to replace glutamine under these conditions was investigated. As Table II shows, we found that $l(+)$-glutamic acid also had strong growth-promoting properties for each organism, but the amounts required varied considerably. For five of the nine strains of bacteria tested, $l(+)$-glutamic acid was just as effective as glutamine, while for the other four organisms at least 11 times as much glutamic acid was required for the production of equivalent growth (see Table III).

These results indicated that glutamine and glutamic acid could both be

used by the bacteria for the same purpose or purposes, but with different degrees of efficiency in some cases. It was unlikely that glutamic acid was being converted into glutamine, since the medium contained no source of ammonia other than the amino acids and other organic nitrogen compounds. In such a medium one would not anticipate the equal effectiveness of glutamic acid and glutamine which was found in five cases. Moreover, it was possible to test the likelihood of this mechanism further, for, if the lesser potency of glutamic acid in the other four cases was due to only partial conversion to glutamine, then the addition of a high concentration of ammonium salts would be expected to produce a much greater growth effect.

TABLE III

Relative Growth-Promoting Properties of Glutamine and l(+)-Glutamic Acid

Bacterial growth level (wet cells per cc.)	Ratios of glutamic acid to glutamine required to produce same bacterial growth levels								
	S. lactis 125	S. lactis UT	S. lactis 7963	S. lactis 374	S. lactis R	S. lactis R8043	L. pentosus 124.2	L. arabinosus 17.5	L. casei ε
γ									
30	25			21					
50	13		20	34	1.4	1.1	1.7	1.0	1.1
70	11		19	54	1.1	1.0	1.5	1.0	2.3
100	11	23	21		1.1	0.9	1.1	1.0	
140	13	15	23		0.9	0.8	1.0	1.0	
180	24	14			0.8	0.7	0.9	0.9	
220		13			0.8	0.7	0.9	0.9	
280		13							
340		13				\			
400		13							

This was tested by comparing the growth action of l(+)-glutamic acid in the presence of 1 mg. of ammonium chloride per ml. with that in the absence of ammonium salts. It was found that the presence of the salt led to a somewhat greater potency of glutamic acid, but the maximum effects produced corresponded to the conversion of only 5 to 20 per cent of the acid to glutamine. Furthermore, it is probable that at least part of this stimulation is of a general nature, since experiments run simultaneously with glutamine on the ammonium chloride medium also showed cases of slight (lesser) growth stimulation. It was therefore concluded that these bacteria do not depend on the conversion of glutamic acid to glutamine for the utilization of the former compound.

It should be mentioned that in the absence of glutamine and glutamic acid, no growth effect by the addition of ammonium chloride alone to the

medium was observed at any time. This has additional interest, since Vickery has shown (9) that green plants can synthesize glutamine from ammonium salts and some carbohydrate metabolite. The lactic acid bacteria tested here showed no signs of such synthesis in the presence of ammonium chloride, glucose, and the other substances of the medium.

The simplest explanation for the growth effects of glutamine and glutamic acid appeared to us to be that they were required for the synthesis of essential cell proteins. The universal distribution of glutamyl units in proteins of different types (10) makes it seem very reasonable that we should expect to find them also in the proteins of these bacteria. The many studies of the rôle of the amino acids in nutrition ((10) chapter 18) all tend to reinforce the theory that protein synthesis occurs by the combination of amino acids. Since we have found that these bacteria are incapable of producing their own glutamic acid or glutamine on the medium provided, it appears logical to consider that the addition of these substances permitted the synthesis of essential proteins, thus producing the observed growth effects.

Further evidence on this point was provided by experiments wherein the yields of protein from known amounts of glutamine and glutamic acid were estimated. It was found that 19 to 20 γ of dry cells of *Streptococcus lactis* 125 were obtained per microgram of glutamine, and 11 γ of dry matter per microgram of $l(+)$-glutamic acid in the case of *Lactobacillus arabinosus* 17.5. If we assume that 50 per cent of the dry matter consisted of protein, then complete utilization of the glutamine and glutamic acid would correspond to the construction of proteins containing 10 to 20 per cent of glutamyl units. These values are in line with the actual glutamic acid contents found for many proteins (10), and the important feature of this calculation is that the order of magnitude of the factor requirements is shown to be compatible with the idea of protein synthesis. The fact that the glutamine or glutamic acid requirements for the bacteria were never found to be below this order of magnitude is further indication of this mechanism.

It is highly interesting that all the organisms tested are apparently capable of utilizing glutamine at what appears to be maximum efficiency, but vary considerably in so far as glutamic acid is concerned. For the synthesis of proteins, this may be taken to indicate the unequal distribution of at least two types of proteinases, which differ in their abilities to use glutamine and glutamic acid. Evidence has accumulated (11) to show that the carboxyl groups in proteins are found in both the free and the amide forms, and the proportions may be different with different proteins. It may be possible to correlate the results found here and the growth of bacteria in the presence of glutamic acid or glutamine with the presence of

glutamyl units in the free acid or amide form in the corresponding proteins produced.

There are of course other functions which glutamine and glutamic acid may perform in connection with the growth of these bacteria, and it is highly possible that protein synthesis is only part of the story. For example, these substances may be essential catalysts in the metabolism of carbohydrate, possibly via oxidative deamination to α-ketoglutaric acid which can act through the citric cycle (12). However, in such a case it would be difficult to understand why aspartic acid and asparagine, which could presumably be converted to oxalacetic acid in similar fashion, were completely without action when tested here. From the work of Krebs and Cohen (13), it appears that the system glutamic acid $\rightleftharpoons$ α-iminoglutaric acid can act catalytically in hydrogen transport, but in the present work it would be difficult to explain the different growth effects of glutamic acid and glutamine on this basis. Other functions of these compounds have been reported in the literature, but we feel that none of them yields as satisfactory an explanation for the growth effects observed here as does the idea of simple requirement for protein synthesis.

SUMMARY

1. Nine lactic acid-producing bacteria are shown to require either glutamine or glutamic acid for growth.

2. Five of these organisms responded as well to glutamic acid as to glutamine, but the other four required at least 11 times as much acid as amide to produce the same effect.

3. It is unlikely that glutamic acid functions through the amide, because both forms are equally potent on an ammonia-free medium in some cases, and the addition of a high concentration of ammonium chloride produces only a minor increase in the effectiveness of glutamic acid.

4. None of the bacteria tested is capable of producing glutamine from glucose and ammonium salts.

5. The most likely mechanism is that the bacteria require glutamine or glutamic acid simply for the construction of cell proteins, particularly in view of the fact that the requirements of these amino acids are of the order of magnitude which would be expected for this function.

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A SIMPLE TISSUE LIQUEFIER

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In the course of studies requiring extractions of tissue, a small, simple, and highly efficient tissue press was developed. The apparatus, drawn to scale in the accompanying sketch (Fig. 1), consists of a heavy walled, brass, cylindrical shell (*C*), a hardened steel piston (*P*) which fits into the barrel of *C* with a clearance of 0.0005 inch, and a brass receptacle disk (*D*).
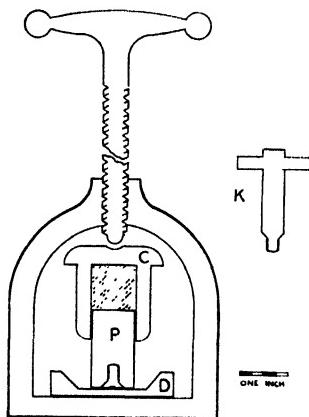
FIG. 1. Tissue press

A key (*K*) is used for removing the piston from the cylinder after the tissue is pressed. Pressure is applied with a screw stirrup clamp.

The tissue to be liquefied is placed in the cylindrical shell, the piston set into the barrel, and the two parts inverted and set on the disk as shown in Fig. 1. It is convenient to place a piece of waxed paper or parchment under the piston; this facilitates the removal of the expressed tissue. The screw is then turned; this applies pressure on the cylinder and forces the tissue out between the piston and the cylinder wall. The piston can be removed from the cylinder by taking out the flat-head machine-screw and attaching the key (*K*) in place.

The clearance between piston and cylinder is so small (0.0005 inch) that the expressed tissue has the consistency of a heavy fluid. It has a smooth even texture, and is so liquefied that it can be drawn into a fine pipette with ease.

The press has been used with various parenchymatous organs, liver, spleen, kidney, testes, and the like. Microscopic examination of the tissue smears, or saline suspensions, revealed no basic tissue architecture. Whole cells are found, separate and distinct, but only rarely are they found in sizable clusters; with kidney, tubules may be recognized but these are distorted and broken into small fragments.

The apparatus can be made as a single unit by fixing the piston permanently to the base-plate of the stirrup clamp, and by fixing the cylinder cup to the screw. The entire unit can then be arranged to work in a horizontal position, and the expressed tissue substance received in a tray or cup below the press. The press as described is used for 10 to 15 cc. of tissue.

ON THE COLORIMETRIC METHOD FOR THE DETERMINATION OF THE K VITAMINS

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Recently a colorimetric oxidation-reduction method (1) for the determination of the K vitamins was described and certain criteria of specificity were outlined. The vitamin present is reduced catalytically and the resulting hydroquinone is treated with an excess of 2,6-dichloroindophenol in the absence of air. The diminution in the color of the indophenol is a measure of the vitamin K originally present. The specificity of the method depends upon the solubility properties of the vitamin, and upon the fact that the oxidation-reduction potential of the substance measured must fall within a specified range. A preliminary reductive treatment of the sample with Claisen's alkali (50 gm. of potassium hydroxide in 25 cc. of water diluted to 100 cc. with methanol) converts colored concentrates to essentially colorless solutions and adds much to the specificity of the test, since only polysubstituted hydroquinones which possess fat-soluble groupings pass into the solution which is tested.[1] With certain concentrates, however, it was found that extraneous substances could simulate vitamin K in every respect except one; namely, they reduce the indophenol at a much slower rate, and are, consequently, easily detected in the course of the test. These substances were shown to possess the properties of the tocopherylquinones and their interference in the performance of the test was diminished by restricting the reading time to 3 minutes, in which time the reduction of the indophenol by the vitamin K hydroquinone is complete, whereas the reduction due to the tocopherylhydroquinones is only just beginning. It is clear that, if a sample contains a large amount of vitamin K_1 and small amounts of the tocopherylquinones, the interference of the latter substance will be insignificant, but as the amounts of the tocopherylquinones increase, the error becomes significant, and if the tocopherylquinone content is large, and the vitamin K content is small, then entirely erroneous results will be obtained.

The present communication is concerned with the interference of these slowly reducing substances in the performance of the test.

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[1] 2-Methyl- and 2,3-dimethyl-1,4-naphthoquinone do not pass through this treatment.

Fig. 1 shows the rate of reduction of 2,6-dichloroindophenol by different concentrations of α-tocopherylhydroquinone when the test is carried out as previously described for the K vitamins. Fig. 2 shows similar data obtained with solutions containing both vitamin K_1 and α-tocopherylhydroquinone, a typical, slowly reducing, interfering substance. For a fixed concentration of vitamin K_1 (12.1 γ per cc.) the quantities estimated
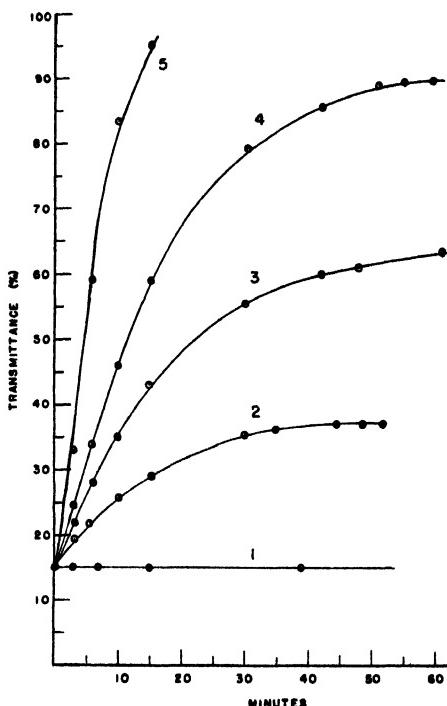
FIG. 1. The reduction of 2,6-dichloroindophenol by tocopherylhydroquinone. Curve 1, control; Curves 2 to 5, obtained with solutions containing tocopherylquinone concentrations equivalent to 3.84, 5.76, 7.70, and 16.0 γ of 2-methyl-1,4-naphthoquinone per cc.

from the 3 minute reading vary from 102 per cent in the presence of 4.81 γ of tocopherylquinone per cc. to 125 per cent in the presence of 19.2 γ of tocopherylquinone per cc. For the solution containing 6.05 γ of vitamin K_1 and 9.62 γ of tocopherylquinone per cc. the apparent recovery from the 3 minute reading is 141 per cent. Thus, with decreasing concentrations of vitamin K_1 and increasing concentrations of the slowly reducing substances, increasingly positive errors are introduced. If, however, the

curves shown in Fig. 2 are extrapolated to zero time, the values so obtained give recoveries of 98, 99, 100, and 100 per cent.

Fig. 3 shows a typical calibration curve obtained with the Evelyn colorimeter with Filter 660. The ordinate is expressed directly in terms of the galvanometer deflections. A straight line is obtained if log $(1/T)$ is plotted against concentration of 2-methyl-1,4-naphthohydroquinone. The initial
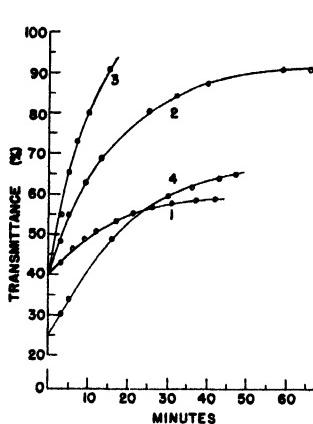
FIG. 2   FIG. 3

FIG. 2. The reduction of 2,6-dichloroindophenol by solutions containing both tocopherylhydroquinone and reduced vitamin K_1. The solutions used to obtain Curves 1, 2, and 3 contained 12.1 γ of vitamin K_1 per cc. (equivalent to 4.40 γ of 2-methyl-1,4-naphthoquinone) in each case, but the tocopherylquinone content was equivalent to 1.86, 3.71, and 7.42 γ of 2-methyl-1,4-naphthoquinone respectively. Curve 4 was obtained from a solution containing 6.05 γ of vitamin K_1 and 9.62 γ of tocopherylquinone, the total being equivalent to 5.90 γ of 2-methyl-1,4-naphthoquinone per cc.

FIG. 3. Calibration curve for the Evelyn photoelectric colorimeter, Filter 660. The abscissae are expressed as 2-methyl-1,4-naphthoquinone in micrograms per cc. of the solution tested. The points obtained are designated as follows: ⊙, solutions of vitamin K_1; ▢, solutions of the 2-methylnaphthoquinone, 3 minute readings; △, solutions of α-tocopherylquinone, final steady readings; circle in a triangle, solutions containing both the tocopherylquinone and vitamin K_1, final readings; ◇ solution of 2,3-dimethyl-1,4-naphthoquinone.
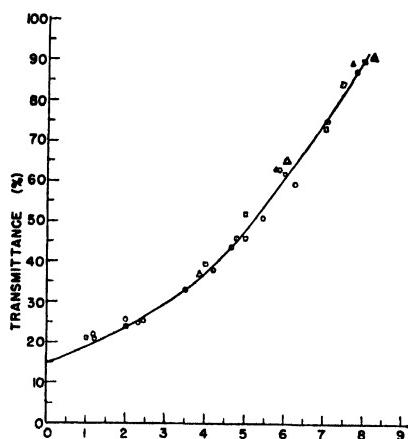
concentration of the indophenol is adjusted to give 15 per cent transmission, and as the indophenol is reduced by increasing concentrations of the hydroquinones, the transmission increases. The abscissa is expressed in micrograms of 2-methyl-1,4-naphthoquinone. The colorimeter was originally calibrated against this substance, and the readings taken 3 minutes after the indophenol and the hydroquinone were mixed. Higher concentra-

tions of the 2-methyl-1,4-naphthoquinone (5 to 8 γ per cc.) give unstable end-points. While the major part of the reduction is complete within 3 minutes, a significant part of the reduction continues beyond this time interval. Since vitamin K_1 gives stable end-points, this material is more suitable for purposes of calibration.

Fig. 3 shows typical data obtained with solutions of 2-methyl-1,4-naphthoquinone or vitamin K_1. The final, steady values obtained with the α-tocopherylquinone (Fig. 1) and the combined values obtained with the solutions containing both vitamin K_1 and tocopherylquinone together (Fig. 2) are also plotted. The most widely divergent points fall within 8 per cent of the theoretical.

It is advantageous to avoid the formation of the tocopherylquinones in the course of the preparation of vitamin K concentrates, and this can be accomplished to a large extent by working under nitrogen at reduced pressures in the absence of light. The use of hydroquinone as an anti-oxidant is entirely feasible, since prodigious quantities of this protective substance are removed by the reductive treatment with Claisen's alkali prior to analysis. But, if a given sample already contains tocopheryl-quinones and other slowly reducing substances, such protective measures are of no value. It seemed desirable, therefore, to investigate means of removing these interfering substances.

Based on the assumption that the interfering substances are largely tocopherylquinones, the method of Tishler and Wendler (2) was used to cyclize these quinones. This reductive ring closure proceeds smoothly, giving excellent yields of the tocopherols, which do not interfere in the test procedure, but vitamin K_1 gave poor recoveries following the same treatment and no optimum could be established. Preliminary reduction of the double bond in the phytyl chain to prevent the possible cyclization of vitamin K_1 did not increase the stability of the vitamin to the treatment reported by Tishler and Wendler. The lability of vitamin K to acids and alkali suggested that duplicate determinations before and after the destruction of vitamin K might give an accurate estimate of the vitamin. Treatment of petroleum ether extracts with 85 per cent sulfuric acid according to the method of Parker and McFarlane (3) removed all of the vitamin K, but all of the slow reducers were simultaneously removed. Destruction of the K vitamins with sodium hydroxide was not standard-ized, since the tocopherylquinones are also destroyed by alkali.

SUMMARY

The influence of extraneous, slowly reducing substances upon the per-formance of the colorimetric test for the determination of the K vitamins

has been investigated. It has been shown that the error caused by this interference is eliminated by extrapolation to zero time.

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THE FORMATION OF PHOSPHORYLATED GLYCERIC ACID IN THE BLOOD CELLS OF VARIOUS SPECIES

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In an earlier paper (1) we reported data showing a great diversity in the concentrations and relative distributions of organic acid-soluble phosphorus compounds in various species of erythrocytes. In most of the mammalian bloods examined the concentration of organic acid-soluble P in the cells was between 50 and 100 mg. per 100 cc., and of this a high proportion was diphosphoglycerate P. On the other hand, only traces of phosphoglycerate were found in the erythrocytes of several ungulates (sheep, goat, deer, beef) and none in the nucleated erythrocytes of birds and reptiles. These findings invited further investigations to determine whether the various species of blood cells differed in their ability to form phosphorylated glyceric acid *in vitro*, and whether the phosphoric esters thus formed in different species of blood might differ in character. It seemed too that such studies should yield evidence concerning the validity of the theory elaborated by Dische (2) concerning the rôle of phosphoglyceric acid as a link in the glycolysis of human and other species of blood.

EXPERIMENTAL

The collection of blood samples and the analytical methods used have been described previously (1). To inhibit the decomposition of phosphoglycerate in the blood sodium fluoride was added to each sample in sufficient quantity to make its concentration 0.02 N. Sodium pyruvate, presumed to be the physiologic hydrogen acceptor in the reaction leading to the formation of phosphoglycerate (2), was added to make its concentration 0.01 N. To blood containing nucleated erythrocytes KCN was added to make its concentration 0.003 N, in order to establish maximal glycolysis and to inhibit the oxidative removal of intermediary substances formed in the breakdown of glucose. All blood samples were incubated at 37.5° for 2½ hours.

Results

The data presented in Table I indicate that phosphorylated glyceric acid accumulated in all the bloods studied, including those which normally do not contain any demonstrable phosphoglycerate. This suggests that

in each blood the breakdown of carbohydrate took place with the intermediate production of phosphoglyceric acid according to the reactions described by Dische (2) as occurring in human blood. Presumably the phosphoglycerate accumulated because the addition of sodium fluoride inhibited its decomposition by interrupting the glycolytic cycle at this stage.

TABLE I

Formation of Phosphorylated Glyceric Acid in Blood Cells of Various Species

Blood + sodium fluoride + sodium pyruvate; analyses were made before and after incubation for $2\frac{1}{2}$ hours at 37.5°.

Species		Whole blood inorganic P	Cells		
			Easily hydrolyzable P	Phosphoglycerate P*	Increase of phosphoglycerate P
		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Pig	Before	7.3	11.9	43.7	
	After	8.6	9.4	49.1	5.4
Rabbit	Before	3.5	13.0	45.2	
	After	2.5	6.0	55.8	10.6
Man	Before	3.8	11.0	26.0	
	After	3.0	7.0	33.0	7.0
Horse	Before	2.1	2.5	33.4	
	After	1.8	1.8	35.6	2.2
Sheep	Before	6.5	5.8	<0.8	
	After	5.2	3.8	3.2	>2.4
Beef	Before	6.7	3.9	<0.7	
	After	5.1	2.7	4.8	>4.1
Pigeon	Before	5.5	18.2	<1	
	After	17.2	1.0	6.3	>5.3
Turkey	Before	5.2	11.2	<1.5	
	After	10.4	0	7.5	>6.0
Chicken	Before	2.4	7.0	<1	
	After	3.8	0.6	6.2	>5.2
Turtle	Before	7.2	11.1	<2	
	After	7.8	2.1	8.6	>6.6

* It was assumed that diphosphoglycerate was formed in the blood of pig, rabbit, man, and horse, and that monophosphoglycerate was formed in the other bloods.

The evidence for the existence of a glycolytic system in the erythrocytes of pig blood is especially significant, because these cells have been held to have no glycolytic power. It is to be noted that in this blood the esterification of inorganic P appeared to be absent, although it was observed in all other mammalian bloods. This might be interpreted as indicating either a preponderant influence of phosphatases, or a weak system for the esterification of adenylic acid with inorganic P. The weak glycolytic

power of these cells favors the second explanation, but both may be valid. Either circumstance would make for instability of the glycolytic system of the pig blood cells.

In blood containing nucleated erythrocytes increases of inorganic P as well as of phosphoglycerate occurred during the incubation period, and the easily hydrolyzable fraction decreased more than in mammalian blood. These observations are in accord with the experiences of Engelhardt (3) who found that adenosine triphosphate in avian blood cells was preserved only if aerobic rephosphorylation of adenylic acid took place. In the experiments reported here the increase of inorganic P was less than the decrease in the concentration of the easily hydrolyzable fraction, indicating that phosphorus was transferred from the easily hydrolyzable fraction to one more resistant to acid hydrolysis, most likely phosphoglycerate.

Results of previous experiments indicated that diphosphoglycerate accumulated in rabbit blood to which pyruvate and sodium fluoride had been added. Hevesy and coworkers (4), using radioactive P as a tracer, established the fact that diphosphoglycerate was continuously formed and decomposed in the blood cells of rabbits. Thus it appeared that diphosphoglycerate is a physiologic intermediary in the glycolytic cycle of rabbit blood cells, and presumably of other cells that have been found to contain this substance. In the case of blood which normally contains little or no phosphoglycerate, however, it was of interest to determine whether the phosphoglycerate formed *in vitro* (as shown in Table I) was the mono- or diphosphoric ester. Beef blood was chosen as representative of mammalian blood with low phosphoglycerate, and goose blood as representative of avian blood with nucleated erythrocytes which normally contain no phosphoglycerate. The phosphoglycerate formed in these bloods in the presence of added pyruvate and fluoride (and KCN, in the goose blood) was isolated in pure form for analysis, as follows:

Isolation of Barium Monophosphoglycerate from Beef Blood—To 1800 cc. of beef blood were added 180 cc. of 0.2 N NaF solution and 60 cc. of sodium pyruvate solution. After incubation at 37.5° for 3 hours, 60 cc. of 10 per cent $CaCl_2$ solution were added and the proteins were precipitated by adding 600 cc. of 40 per cent trichloroacetic acid. The semiliquid mass was centrifuged and the supernatant fluid decanted and filtered. The clear filtrate was made alkaline with 30 per cent NaOH solution and then reacidified with nitric acid to pH 3. A slimy precipitate that formed, consisting mainly of CaF, was removed by filtering, and 50 cc. of a 25 per cent solution of lead acetate, at pH 4, were added to the filtrate. After standing overnight the resultant precipitate was centrifuged, washed twice by centrifugation with approximately 0.2 per cent lead acetate, and then

decomposed with hydrogen sulfide. The precipitated lead sulfide was suspended in dilute nitric acid and H_2S gas again passed through it. The combined filtrates were adjusted to pH 3 and treated with a few cc. of 20 per cent mercuric acetate solution in 2 per cent acetic acid to precipitate the nucleotides. The bulky precipitate was removed by centrifugation and the excess of mercury in the supernatant fluid was removed by bubbling H_2S gas through it. Following this the solution was aerated. The inorganic P⁻ was then precipitated with magnesium nitrate in ammoniacal solution at pH 10, and removed by filtering. The filtrate was reacidified with acetic acid to pH 6, and an equal volume of alcohol was added, followed by 100 cc. of 25 per cent lead acetate solution. The resulting precipitate was centrifuged the next day, washed repeatedly by centrifugation with weak lead acetate solution, and then decomposed with H_2S. The precipitate of lead sulfide was washed, and the filtrate and washings were combined and aerated to remove the excess of H_2S. The last traces of nucleotides were precipitated with mercuric acetate at pH 6. This small amount of precipitate was removed, the excess mercury in the solution precipitated with H_2S, and the filtrate was aerated.

The solution was adjusted to pH 6, and 5 cc. of a 50 per cent solution of barium acetate and 2 volumes of alcohol were added. A flocculent precipitate appeared. This precipitate was repeatedly washed by centrifugation with 65 per cent alcohol, redissolved in a small volume of 0.03 N HCl, and alcohol was slowly added. A precipitate appeared, at first amorphous and then becoming crystalline after the container was scratched with a glass rod. It was washed with alcohol and ether and dried. The weight was 110 mg. A preliminary analysis indicated the presence of the barium salt of monophosphoglyceric acid. It was recrystallized and dried *in vacuo* at 58°.

$C_3H_5O_7PBa \cdot 2H_2O$. Calculated. Ba 40.5, P 9.13, glyceric acid 30.2
                Found.   " 40.2, " 9.21,   "   " 30.6

Isolation of Barium Monophosphoglycerate from Goose Blood—12 cc. of 0.2 N NaF solution, 6 cc. of 0.2 N sodium pyruvate solution, and 6 cc. of 0.05 N KCN solution were added to 120 cc. of goose blood, and the mixture was incubated 3 hours at 37.5°. The proteins were precipitated with trichloroacetic acid and removed by centrifugation and filtration. From the filtrate inorganic P and phytic acid (5) were precipitated with magnesium acetate and ammonium hydroxide at pH 10. The precipitate was dissolved and similarly reprecipitated in a small volume. Lead acetate was added to the combined supernatant fluids, adjusted to pH 7, and the lead was removed from the resultant precipitate as described above. The bulk of the nucleotides was precipitated with mercuric acetate at pH 3,

the excess of mercury was removed, and barium acetate then added at pH 6. The resultant precipitate, consisting mainly of BaF, was removed and the phosphoglycerate was precipitated with 2 volumes of alcohol. This precipitate was dissolved in 0.05 N HCl, the last traces of nucleotides were removed with mercuric acetate at pH 6, and the phosphoglycerate was reprecipitated as the barium salt. This salt was washed several times, re-dissolved in a small volume of 0.03 N HCl, and then obtained in a crystalline form by the cautious addition of 1.5 volumes of alcohol. It was recrystallized once more from a small volume of fluid, washed with alcohol and ether, and dried *in vacuo* over phosphorus pentoxide. The yield was 3.4 mg.

C₃H₅O₇PBa·2H₂O

$C_3H_5O_7PBa \cdot 2H_2O$
- Calculated. P 9.13, glyceric acid 30.2, P to glyceric acid ratio 1:1
- Found.   " 7.9,   "   " 26.8, " "   "   "   " 1:1.03

The low values for P and glyceric acid appear to be due to contamination with BaF. The ratio of phosphorus to glyceric acid seems to rule out the presence of any other phosphoric ester in the preparation, including diphosphoglyceric acid.

DISCUSSION

Under the conditions of these experiments the concentration of phosphorylated glyceric acid increased in all the bloods studied, as was expected if the breakdown of glucose occurred according to the Embden-Meyerhof theory. Also, in all mammalian bloods with the exception of pig blood, inorganic P was esterified in the presence of pyruvate and sodium fluoride, indicating the general occurrence of the esterification of inorganic P coupled with the formation of phosphoglyceric acid as observed by Dische (2). In avian nucleated erythrocytes, and in the erythrocytes of mammals which contain little if any phosphoglycerate, monophosphoglyceric acid is formed in the glycolytic cycle, as it is in all other tissues thus far studied; whereas in the erythrocytes of most mammals which normally contain considerable amounts of diphosphoglycerate the latter substance is formed as the physiologic intermediary. Recent studies from Warburg's laboratory may furnish a better understanding of the mechanism of the formation of mono- and diphosphoglyceric acid in blood cells and of the relationships between these two compounds. Using purified enzyme preparations from yeast, Negelein and Broemel (6) found a labile diphosphoglyceric acid to be formed as a precursor of monophosphoglyceric acid. This labile ester differed in structure from the stable 2,3-diphosphoglyceric acid of blood cells. Monophosphoglyceric acid was produced from the labile ester by dephosphorylation. Such findings suggest that in blood cells which normally have a high content of diphosphoglyceric acid, the labile isomer

is transformed into the stable compound, whereas in other blood cells dephosphorylation of the labile ester with formation of monophosphoglyceric acid takes place.

Differences in the concentrations of phosphorylated glyceric acid in blood cells possibly may be correlated with differences between mono- and diphosphoglycerates in their susceptibility to enzymatic attack. Diphosphoglyceric acid is known to be very slowly attacked by phosphatases and to require intact cells for its decomposition (7). Monophosphoglyceric acid, on the other hand, is rapidly decomposed in hemolysates (8). Thus it may be that because of the different rates of enzymatic attack the equilibrium between synthesis and decomposition is strongly in favor of breakdown in cells which form monophosphoglyceric acid, compared with that in cells which form diphosphoglyceric acid.

In a previous paper (1) we suggested that the ability to form diphosphoglyceric acid, absent in phylogenetically older blood cells, appeared to be an acquired characteristic of mammalian erythrocytes. It appeared that the low concentration of organic acid-soluble phosphorus in certain species of mammalian cells, correlated with the absence of diphosphoglycerate and the occurrence of monophosphoglycerate as an intermediary instead of diphosphoglycerate in some, might constitute secondary developments of ontogeny. These conclusions are supported by the findings of Green and Macaskill (9) and Malan (10), who found high concentrations of organic acid-soluble P in the blood of lambs and calves. We also have found high concentrations of organic acid-soluble P in the blood cells of calves during the 1st week of life, with phosphoglycerate accounting for half of the organic acid-soluble P (unpublished data). Such findings at least suggest that the blood cells in the young of all mammalian species have somewhat similar high concentrations of phosphorus and a similar ability to form diphosphoglyceric acid.

SUMMARY

As expected according to the Embden-Meyerhof theory, phosphorylated glyceric acid accumulated in each of ten species of bloods during the incubation of these bloods with added sodium fluoride and pyruvate. Under these conditions monophosphoglycerate was formed in beef blood, taken as representative of the group of mammalian bloods with a low concentration of organic acid-soluble phosphorus in the red cells, and in goose blood, representative of avian bloods with nucleated erythrocytes.

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SOME NEW DIETARY ESSENTIALS REQUIRED BY GUINEA PIGS

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Since the development of highly purified diets capable of supporting good growth of rats and mice, it has been considered advisable to search for new dietary essentials with the aid of other species of mammals. The recognition of the essentiality of nicotinic acid (1), pantothenic acid (2), and inositol (3) following the employment of less commonly used species (*i.e.*, dogs, chicks, and mice) was a further reason for the use of a new species. Accordingly, the nutritive requirements of the guinea pig were investigated. In this paper data will be presented to show that besides the water-soluble vitamins now recognized in pure form, the guinea pig requires at least three other dietary essentials for growth and maintenance of life. For convenience these factors have been designated GPF-1, GPF-2, and GPF-3. The concentration of one of these substances to such an extent that only 5 mg. per day were sufficient to produce good growth (400-fold concentration) will be described.

EXPERIMENTAL

General Assay Procedure—Groups of three guinea pigs, each between 1 and 6 days of age (60 to 90 gm.), were placed together in a cage and fed the appropriate diet and water *ad libitum*. In addition, each animal was given a solution of 10 mg. of ascorbic acid each day by pipette. Body weights were taken once each week. The various supplements were evaluated from consideration of the average weekly gain over a 3 week test period, and by the percentage of survival during this period. The observations with each supplement were repeated at least once with a subsequent group of three guinea pigs.

No characteristic symptoms, except failure of growth and death, occurred with sufficient regularity in guinea pigs deficient in GPF-1 or GPF-2 to permit use of such symptoms as criteria of assay. Before animals died from a deficiency of either of these vitamins, they became inactive and very emaciated. In GPF-2 deficiency the hair was observed invariably to stand on end. This made the animals look and feel like cotton. However, rough fur was sometimes observed as a result of a deficiency of GPF-1. A frequent symptom in the later stages of deficiency of GPF-1 was the inability

* With the technical assistance of A. G. C. White.

to stand. The animals sometimes remained on one side, limp and helpless, for several days before death.

Response to Highly Purified Ration—A basal ration was prepared by mixing the following substances: sucrose 76 gm., vitamin-free casein 18 gm., salts (4) 5 gm., fortified corn oil 1 gm.,[1] choline 100 mg., inositol 100 mg., thiamine 0.2 mg., riboflavin 0.5 mg., pyridoxine 0.2 mg., nicotinic acid 1 mg., pantothenic acid 1 mg., and ascorbic acid 10 mg. When guinea pigs were fed this ration, they failed to grow and died in from 2 to 3 weeks. A typical response in growth is illustrated by the first entry in Table I. It was evident that a diet which was sufficient for the rat and the mouse was totally inadequate for the guinea pig.

TABLE I

Evidence for Two New Factors

The basal ration was the highly purified mixture described in the text. The dried grass was incorporated as 2 per cent of the ration. The numbers in the second column refer to the percentage of the supplement not including the dried grass.

Supplement	Amount in ration	Average weekly gain
	per cent	*gm.*
None......................		7
Dried grass	2	7
Linseed oil meal + dried grass	25	19
	10	14
50% ethanol extract of linseed oil meal + dried grass..	Equivalent to 25	3
50% ethanol residue of linseed oil meal + dried grass	" " 25	5
Above extract + residue + dried grass...........	25	24

A supplement of 10 gm. per day of fresh cabbage produced fair growth. However, green and succulent material was not essential, for it was found that the feeding of Steenbock's stock ration[2] plus ascorbic acid resulted in gains of 30 to 40 gm. per week. Therefore the individual constituents of Steenbock's stock ration were tested as supplements to the highly purified basal ration. No single material was adequate when it was fed at the same level as it occurred in the Steenbock ration. However, linseed oil meal produced some response, and so it was tested at higher levels. 25 per cent

[1] To each 100 gm. of corn oil were added 1 gm. (200,000 u.s.p. units) of vitamin A concentrate, 1 gm. of viosterol (10,000 u.s.p. units of vitamin D), 1 gm. of a 40 per cent concentrate of α-tocopherol from wheat germ oil, and 10 mg. of 2-methyl-1,4-naphthoquinone. The vitamin concentrates were generously supplied by Dr. K. Hickman of Distillation Products, Inc.

[2] Composed of corn 76, casein 5, milk powder 50, linseed oil meal 16, alfalfa leaf meal 2, bone ash 0.5, and salt 0.5 parts.

of linseed oil meal produced very good growth for the first 3 to 4 weeks. The animals appeared in excellent health and their fur had a smoothness and gloss better than that seen on animals fed standard rations. Results in the following weeks will be presented below under "Evidence for third factor." 10 per cent of linseed oil meal was not quite as good as 25 per cent, and 5 per cent was of little value.

Separation of GPF-1 and GPF-2—When linseed oil meal was extracted three times at room temperature with 50 per cent ethanol, neither extract nor residue would support growth when it was added to the highly purified basal ration. When the two fractions were combined, activity was recovered (Table I). (In an attempt to supply the so called grass juice factor (5), 2 per cent of a specially dried grass (Cerophyl) was added to the purified basal ration in this and subsequent tests.)

The substance extracted by 50 per cent alcohol has been designated GPF-1, and that insoluble in this solvent has been called GPF-2.

Basal Ration for Assay of GPF-2—To produce a ration for the assay of GPF-2 each 100 gm. of the highly purified basal ration described above were mixed with 2 gm. of dried grass (Cerophyl) and the extract obtained by extracting 25 gm. of linseed oil meal three times with 300 cc. portions of 50 per cent ethanol. The extract was concentrated under reduced pressure to about 5 cc. before it was added to the other ingredients of the ration.

Only preliminary studies have been made of GPF-2. It was not extracted by alcohol, acetone, or acetone and HCl. Besides linseed oil meal, 10 per cent of dried grass (Cerophyl) or 10 per cent of the alcohol-insoluble portion of aqueous extract of beef liver also contained adequate amounts of this factor. It was not replaceable by flaxseed mucilage,[3] calcium gluconate, gum arabic (7), glucurone,[4] chondroitin,[5] mucic acid, galactose, biotin, or *p*-aminobenzoic acid. Since these substances were inactive, it is not necessary to present the hypotheses which led to their trial. The data are summarized in Table II.

Basal Ration for Assay of GPF-1—The basal ration for the assay of GPF-1 was produced by mixing each 100 gm. of the highly purified basal ration described above with 2 gm. of dried grass (Cerophyl) and the residue left after 25 gm. of linseed oil meal had been extracted with 50 per cent ethanol.

Concentration of GPF-1—A few properties of GPF-1 which may be of value in the concentration of it will be enumerated. Other properties will

[3] Part of this material was given by the Archer-Daniels-Midland Company of Minneapolis; part was prepared according to Neville (6).
[4] Kindly supplied by Dr. W. F. Goebel of the Hospital of The Rockefeller Institute for Medical Research.
[5] Kindly supplied by Dr. David Klein of The Wilson Laboratories.

appear from the manner in which the best concentrate was obtained (see below). It was not extracted from linseed oil meal by 95 per cent ethanol or by acetone. It was not readily destroyed by alkali, for when a crude preparation was allowed to stand for 1 day in 0.10 N barium hydroxide, and then freed of barium with H_2SO_4, part but not all of the activity remained. Fullers' earth adsorbed it, and it was eluted by barium hydroxide or by dilute alcoholic·pyridine. It was only partially removed from acid aqueous solution by norit (20 gm. of norit for the extract from 100 gm. of linseed oil meal).

A typical concentrate was prepared as follows: 1 kilo of linseed oil meal was suspended in 3 liters of 50 per cent ethanol and allowed to stand overnight. The suspension was filtered and the residue was extracted twice more in similar fashion. The combined extracts were concentrated under

TABLE II

Efficacy of Some Materials and Compounds As GPF-2

Supplement	Amount in ration	Average weekly gain
	per cent	*gm.*
None...		3
Dried grass.......................................	10	27
Alcohol-insoluble liver extract	5	27
Linseed mucilage..................................	10	8
Gum arabic.......................................	10	6
Calcium gluconate........	5	6
Mucic acid.......................................	10	3
p-Aminobenzoic acid..............................	0.1	5
Biotin..	0.0004	3

reduced pressure to a sirup. This residue was warmed with 1 liter of water and filtered. The filtrate was diluted to 5 liters, acidified (pH 2) with HCl, and stirred with 250 gm. of fullers' earth (Lloyd's reagent). The filtrate was treated again with 100 gm. of fullers' earth. The combined adsorbates were washed with water, then eluted by suspension in 1 liter of cold, saturated barium hydroxide. The elution was repeated. The eluates were freed of barium with H_2SO_4 and the filtrate from the $BaSO_4$ was treated with excess lead acetate. The filtrate from the precipitate which formed was freed of lead with H_2S and the filtrate from the PbS was concentrated under reduced pressure to a sirup (5 gm.). This material was dissolved in 150 cc. of water and extracted ten times with butanol. The residual aqueous phase (2.5 gm. of dry matter) contained all of the activity of the linseed oil meal in so far as could be determined by the assay method. The data are summarized in Table III.

Evidence for Third Factor (GPF-3)—When both GPF-1 and GPF-2 were added to the basal ration in the form of linseed oil meal, growth was good and the condition of the animals was normal for the first 3 to 4 weeks. Following this, growth ceased abruptly and in many instances loss of weight ensued. Death resulted within a week or two. At the time that growth ceased, the animals lost their sleek appearance but they usually did not appear rough or moribund until just before death. Dried grass (Cerophyl) as 5 per cent of the ration reduced but did not eliminate this condition. It was for this reason that grass was added to the basal rations for assay of GPF-1 and GPF-2. No material was found which would eliminate the symptoms when it was added to the ration in relatively small amounts. For this reason there is no certainty that a dietary defi-

TABLE III ᐧ

GPF-1 Activity of Fractions Prepared from Linseed Oil Meal

The amount of fraction derived from 25 gm. of linseed oil meal was added to 100 gm. of basal ration.

Supplement	Average weekly gain
	gm.
None............	5
Water-soluble portion of 50% ethanol extract of linseed oil meal .	24
Above, Ba(OH)$_2$-treated...	16
Norit filtrate.	17
Fullers' earth filtrate.	5
95% ethanol extract of linseed oil meal	2
Ba(OH)$_2$ eluate of fullers' earth	23
Ethanolic pyridine eluate of fullers' earth	22
Lead acetate filtrate..	32
Butanol residue..	27

ciency was involved. However, since the condition was not seen when either Steenbock's stock ration or the usual type of standard laboratory ration for guinea pigs was fed, it was felt that a deficiency was involved.

DISCUSSION

The data presented above show that guinea pigs require three or more dietary essentials besides those which are sufficient for the growth of species such as rats and mice. Neither of the two of these factors which were studied in any detail was identical with substances which have been shown to possess vitamin activity for other animals. It is not known whether any of the factors are identical with unidentified and unpurified factors required by chicks (7–11). GPF-1 bears some relationship to the factor described by Stokstad *et al.* (8) as necessary for chicks. The question of the identity

of these new factors with those required by other organisms must await
isolation of the substances in pure form. Considerable progress in purifi-
cation of one of the new vitamins has already been made, and work is con-
tinuing on further concentration.

SUMMARY

Guinea pigs did not grow and soon died when they were fed a ration
composed of casein, sucrose, inorganic salts, corn oil, vitamins A, D, K, E,
thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, choline,
inositol, and ascorbic acid. Two new factors, one soluble in 50 per cent
alcohol (GPF-1), and one insoluble in this solvent (GPF-2), have been
shown to be necessary for the survival and growth of young guinea pigs.
A third factor seemed to be necessary for continued growth and life of the
animals for periods longer than a few weeks. Several properties of GPF-1
have been studied and advantage has been taken of these in the concentra-
tion of this factor. Purification of GPF-1 has been carried to a point such
that 5 mg. per day produced good growth. Several compounds which
have recently been found essential for chicks did not replace GPF-2.

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THE FRACTIONATION OF THE AMINO ACIDS OF TOBACCO MOSAIC VIRUS PROTEIN

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The determination of some of the amino acids of tobacco mosaic virus protein by colorimetric and other special methods has been described in preceding papers (1, 2). Since plants diseased with tobacco mosaic contain rather large amounts of the virus protein (3), it is possible to obtain sufficient quantities of the substance to permit the fractionation and subsequent isolation of many of the amino acids for which simpler methods of analysis are not yet perfected. It is the purpose of this report to present the results of the application of isolation methods to hydrolysates of tobacco mosaic virus. The values reported are minimum ones, for losses unavoidably occur during the many steps involved and in many cases only partial precipitation was obtained. It was not possible to complete the separation and analysis of all of the fractions obtained. Nevertheless, the results are presented, for they provide further information concerning the amino acids present and indicate to some extent the fractions in which the remainder may be found. A preliminary report of a portion of the work has been presented (4) previously.

In Table I are given analytical values obtained from a variety of sources. The data in the second column are, with one exception, the maximum figures that have been reported. Since Knight and Stanley (8) used an improved method for phenylalanine, the value obtained by them is given. The other values reported in the fourth column are not necessarily conflicting, for it is to be expected that, in any procedure in which fractionation and isolation are resorted to, low results will be obtained.

EXPERIMENTAL

Preparation of Protein—The sample of tobacco mosaic virus used was the same as the chemically prepared sample previously described (1, 2). Since it contained the same amounts of cysteine, tyrosine, tryptophane, and arginine as did the ultracentrifugally isolated preparations, it was considered to be essentially pure virus protein. The values reported were corrected for the 8 per cent moisture that the sample contained.

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Preparation of Hydrolysates—Two separate portions of the sample, each of about 100 gm., were hydrolyzed by being boiled for 18 hours with 1000 ml. of 20 per cent HCl. Independent tests had shown that the amino nitrogen reaches a maximum value in that time. Excess HCl was removed by repeated concentrations under reduced pressure. The residues

TABLE I

Composition of Tobacco Mosaic Virus

Component	Per cent by weight*	Bibliographic reference No.	Other values
Alanine........................	2.4	†	
Amide nitrogen......	1.9‡	†	1.8%‡§
Arginine..	9.0	(1)	8.8%†
Aspartic acid	2.6	†	2.3%†
Cysteine	0.7	(2, 5)	
Glutamic acid	5.3	†	3.3% (6)
Glycine	0.0	(1)†	
Histidine.	0.0	(1)†	
Leucine....	6.1	†	4.9%,† 0.85% (6)
Lysine	0.0	(1)†	
Methionine	0.0	(2)	
Nucleic acid.	5.8	(7)	
Phenylalanine. .. .. .. '.	6.0	(1, 8)	2.1%†
Proline...	4.6	(1)	2%,† 3.8%†
Serine.....	6.4	(1)	1.4%†
Threonine.	5.3	(1)	
Tryptophane	4.5	(1, 8)	
Tyrosine	3.9	(1)	3.8% (8), 1.4%,† 1.1% (6)
Valine.......	3.9	†	
Total	68.4		

* All values for the percentage of amino acids are uncorrected for the 1 molecule of water added per molecule of amino acid during hydrolysis.
† See "Experimental."
‡ Calculated as ammonia.
§ Dr. G. L. Miller, personal communication.

were dissolved in water and filtered, and the insoluble humin was washed thoroughly with water. In each case the filtrate and washings were combined and diluted to 1000 ml. These were designated Hydrolysates A and B, respectively.

Aliquot portions were taken from each for Kjeldahl determinations. Other portions were used for the determination of ammonia, acid-soluble humin, and amino nitrogen by the Van Slyke procedure (9). The results

of these analyses are presented in Table II. The values for ammonia nitrogen agree well with that obtained by Miller;[1] namely, 1.5 per cent amide nitrogen, equivalent to 1.8 per cent when calculated as ammonia. The method used by Miller was based on the findings of Shore et al. (10). The amide nitrogen was calculated from data on the rate of ammonia formation.

TABLE II

Humin, Ammonia, and Amino Nitrogen in Acid Hydrolysate

Fraction	Amount		N in fraction	
	Hydrolysate A	Hydrolysate B	Hydrolysate A	Hydrolysate B
	gm.	*gm.*	*gm.*	*gm.*
Protein, oven-dry............	92.29	93.66	14.674*	14.891*
Filtered hydrolysate.........			14.542	14.507
Acid-insoluble humin..	2.394	2.026	0.135	0.131
Ammonia........................	1.771	1.776	1.459†	1.463‡
Amino N.................			9.585§	10.000‖

* 15.9 per cent N.
† 9.94 per cent of total N; 1.92 per cent of protein, calculated as ammonia.
‡ 9.82 per cent of total N; 1.81 per cent of protein, calculated as ammonia.
§ 65.4 per cent of total N in filtered hydrolysate.
‖ 68.9 per cent of total N in filtered hydrolysate.

Hydrolysate A

Basic Amino Acids—The basic amino acids were precipitated by the method of Vickery and Leavenworth (11) after dilution as recommended by Vickery and Shore (12). The histidine fraction contained but 2.7 mg. of nitrogen and yielded no flavianate. Similar fractions had previously given negative Pauly tests (1). The lysine fraction contained 37 mg. of nitrogen and, while a few picrate crystals were obtained, they were not regained upon recrystallization. 19.548 gm. of recrystallized arginine flavianate were obtained. By colorimetric determinations (13) it was found that 1.189 gm. of arginine escaped precipitation. The total corresponds to an arginine content of 8.9 per cent of the virus protein, a value in fair agreement with that previously reported (1).

Dicarboxylic Acids—The various filtrates obtained during the separation of the basic amino acids were freed of added substances by the methods used by Jukes (14). They were combined and concentrated under reduced pressure to about 400 ml. The dicarboxylic acids were precipitated twice with barium hydroxide and alcohol according to the directions of Jones

[1] Dr. G. L. Miller, personal communication.

and Moeller (15). Barium was removed, the fraction concentrated to 100 ml., and aspartic acid precipitated as its copper salt. The filtrate and washings were freed from copper and concentrated under reduced pressure to 100 ml. Following saturation with HCl gas, the solution was refluxed for 5 hours and then saturated again with HCl gas at 0°. After 3 days at 3°, the precipitated glutamic acid hydrochloride was filtered off. A second crop was obtained by concentrating the filtrate and washings to 30 ml. and repeating the precipitation with HCl. The two crops weighed 6.33 gm., corresponding to 5.5 per cent glutamic acid in the virus protein. Following recrystallization, the salt contained 7.83 per cent N, melted at 210°, and in 20 per cent HCl $[\alpha]_D = +31.7°$, calculated as the free acid. The corresponding constants for glutamic acid hydrochloride are 7.65 per cent, 210° (16), and +31.6°, respectively. Only the usual naturally occurring optical isomer could be isolated, even when the hydrochloride was precipitated directly from the hydrolysates of other samples.

The filtrate from the precipitation of glutamic acid of Hydrolysate A was freed from chloride, concentrated to 40 ml., treated with $CuCO_3$, and a second crop of copper aspartate obtained. The two crops weighed 4.719 gm., corresponding to an aspartic acid content of 2.4 per cent of the virus. The salt contained 5.62 per cent N (calculated for $C_4H_5O_4NCu \cdot \cdot 3H_2O$, 5.62 per cent N). Copper was removed with H_2S and the free acid was crystallized from dilute alcohol. It contained 10.6 per cent N (calculated 10.5 per cent).

The filtrate was again freed from copper with H_2S and examined for the presence of hydroxyglutamic acid. No evidence of its presence was obtained. The fraction still contained 806 mg. of nitrogen, 46 per cent of which was not extractable with butyl alcohol. It seems probable, therefore, that considerable amounts of the dicarboxylic acids escaped precipitation. The values reported are consequently probably much too low, but further attempts to precipitate these amino acids were unsuccessful. The amide nitrogen data indicating a dicarboxylic acid content of about 15 per cent also suggest incomplete precipitation.

The butyl alcohol-soluble portion of the above fraction was combined with the main filtrate from the precipitation of the dicarboxylic acids. The solution was acidified with sulfuric acid, and the alcohol was removed by distillation. Subsequently, the sulfuric acid was quantitatively removed with barium hydroxide. The solution was then concentrated to 250 ml. and tyrosine allowed to crystallize out at 0°. A second crop was obtained after a further concentration. The crude tyrosine was recrystallized and, when compared colorimetrically with pure tyrosine, appeared to be 99 per cent tyrosine. It weighed 1.3 gm., corresponding to 1.4 per cent or less than half of the total amount in the virus (1).

Conversion to Copper Salts—The combined filtrates from the tyrosine crystallization, after removal of added substances, were concentrated to 200 ml. and extracted with butyl alcohol. The aqueous phase contained 1.08 gm. of N, 78.5 per cent of which was in the amino form. An unsuccessful attempt was made to isolate threonine and serine from this fraction by the method used by Woolley and Peterson (17). Colorimetric estimation of serine (18) indicated that this fraction contained 1.3 gm. of serine. Nothing further was learned of the nature of the amino acids in this fraction other than that 83 per cent of the nitrogen was found in the copper salt fraction soluble in ethyl alcohol, 90 per cent of which was amino nitrogen.

The butyl alcohol fraction was evaporated to dryness by distillation under reduced pressure. The residue, when dissolved in water, contained 5.09 gm. of N, 90.5 per cent of which was in the amino form. The amino acids were converted to their copper salts and extracted successively with water and absolute methyl alcohol by the method described by Brazier (19).

Methyl Alcohol-Soluble Copper Salts—Methyl alcohol was removed by distillation under reduced pressure and the copper removed with H_2S from an aqueous solution. The solution of amino acids was evaporated to dryness under reduced pressure, then dried *in vacuo* over P_2O_5 at 37°. The residue was extracted with five 500 ml. portions of absolute ethyl alcohol. The insoluble residue, when dissolved in water, contained 2.1 gm. of nitrogen. Fractional crystallization with ethyl alcohol and water resulted in the isolation of 3.6 gm. of valine, containing 11.97 per cent N (valine = 11.96 per cent N), corresponding to 3.9 per cent of the virus. The *p*-nitrophenylhydrazone of the volatile aldehyde obtained by oxidation with chloramine-T melted at 131°. The *p*-nitrophenylhydrazone of isobutyraldehyde has a melting point of 131–132°.

Alcohol was removed from the alcohol-soluble fraction and the residue dissolved in 250 ml. of water. It contained 1.093 gm. of N, of which 0.618 gm. was non-amino nitrogen. To 10 ml. portions acidified with HCl, ammonium rhodanilate dissolved in methyl alcohol was added in small portions and the precipitate which formed was examined under the microscope. The point at which a homogeneous precipitate was no longer formed was determined. Then 200 ml. of the fraction were acidified and treated with the requisite amount of the reagent (8.33 gm. in 50 ml. of methyl alcohol). The mixture was stirred for an hour, then placed at 4° for 4 hours. The air-dry precipitate weighed 7.07 gm. and contained 6.1 per cent N (calculated 6.2 per cent for proline rhodanilate). This corresponds to a proline content of 2.02 per cent of the virus, a value much lower than that previously reported, 4.6 per cent (1), indicating that serious losses were suffered.

Water-Soluble Copper Salts—Copper was removed with H_2S. The

filtrate and washings were concentrated under reduced pressure to 500 ml. The fraction contained 728 mg. of nitrogen. It was not found possible to precipitate glycine by means of potassium trioxalatochromiate (20). Attempts to precipitate alanine with sodium dioxpyridate (21) were also unsuccessful.

Copper Salts Insoluble in Water and in Methyl Alcohol—The copper was removed as previously described. Phenylalanine was estimated by means of the Kapeller-Adler test (22). The fraction contained 1.96 gm., corresponding to 2.1 per cent of the virus. Tests with Folin's (23) phenol reagent indicated that the fraction contained 143 mg. of tyrosine, 52 mg. of which were obtained by crystallization from a small volume in the cold room. Further fractional crystallizations yielded several crops of leucine which, when combined and recrystallized, weighed 4.386 gm. and contained 10.7 per cent N (calculated 10.8 per cent). 79 mg. of recrystallized leucine were oxidized with 160 mg. of chloramine-T. The *p*-nitrophenylhydrazone of the volatile aldehyde thus formed melted at 109°. The melting point of the *p*-nitrophenylhydrazone of isovaleraldehyde is 109° (24). The leucine isolated corresponds to 4.9 per cent of the virus.

Hydrolysate B

Basic Amino Acids—A procedure similar to that used for Hydrolysate A yielded no histidine or lysine derivative, but 20.042 gm. of arginine flavianate were obtained. After corrections for solubility are made, this indicates an arginine content corresponding to 8.5 per cent of the virus protein.

Dicarboxylic Acids—The combined filtrates obtained after the removal of the basic amino acids were further concentrated to 200 ml. and extracted for 48 hours with butyl alcohol under reduced pressure in a continuous extractor. The aqueous phase, containing 2.2 gm. of N, was subjected to a Foreman precipitation. The precipitate was redissolved in 175 ml. of water and the precipitation repeated. Calcium was removed from the dissolved precipitate with oxalic acid. Glutamic acid hydrochloride and copper aspartate were isolated as previously described. The yields, calculated as per cent of the virus protein, amounted to 5.1 per cent of glutamic acid and 2.8 per cent of aspartic acid.

Conversion to Copper Salts—The butyl alcohol phase was subjected to the same procedure as was described for the like fraction of Hydrolysate A, except that the copper salts were extracted by the method of Town (25).

Methyl Alcohol-Soluble Copper Salts—After the removal of methyl alcohol and copper, the fraction was tested for hydroxyproline by the method of McFarlane and Guest (26) and for proline by the method of Guest (27). The former test was negative, while the latter indicated the

presence of 3.80 gm. of proline, corresponding to 4.13 per cent of the virus. Proline was isolated from an aliquot portion as the rhodanilate by the method of Bergmann (28). The material was 98 per cent pure when tested by the method developed by Bergmann and Stein (29), and the amount corresponded to a proline content in the virus protein of 3.85 per cent. Three-fifths of the fraction was evaporated to dryness and treated with absolute alcohol. 52.5 per cent of the N was soluble in the alcohol, and from this fraction proline was isolated as the rhodanilate in an amount corresponding to 3.77 per cent of the virus. Hence, fewer losses were encountered in the fractionation of this hydrolysate. The remaining 754 mg. of nitrogen in this fraction were not further identified.

Copper Salts Soluble in Water and Insoluble in Methyl Alcohol—This fraction contained 2.472 gm. of N, an amount considerably larger than was found in the corresponding fraction of Hydrolysate A. This indicates that the two different methods of extracting the copper salts did not result in identical separation. It was thought possible that the fraction may have contained some dicarboxylic acids that escaped precipitation. Consequently, after the solution was freed from copper, an aliquot portion was subjected to precipitation with calcium hydroxide and alcohol. 9 per cent of the nitrogen was precipitated.

Two 40 ml. samples of the original copper-free fraction were acidified with HCl and 4 gm. of sodium dioxpyridate (21) were added to each. The mixtures were stirred for ½ hour, then seeded with *l*-alanine[2] dioxpyridate, and allowed to stand 2 days at 3°. Yields of 1.057 and 1.045 gm. were obtained, corresponding to 2.4 per cent alanine in the virus. The precipitates averaged 2.8 per cent amino N (calculated 2.8 per cent). No glycine could be detected through precipitation with potassium trioxalatochromiate or by colorimetric estimation (30).

Copper Salts Insoluble in Water—The insoluble salts were suspended in water, acidified with sulfuric acid, and treated for 2 hours with H_2S with stirring. The filtrate and washings were concentrated under reduced pressure to 500 ml. The fraction contained 1.232 gm. of N. A 50 ml. sample was evaporated to dryness under reduced pressure and the residue dissolved in 14 ml. of 0.5 N HCl. To the hot solution were added 2 gm. of the sodium salt of 2-bromotoluene-5-sulfonic acid (31).[3] The solution was placed at 3° for 2 days. No precipitate formed but, when the solution was seeded with the *l*-leucine salt of the same sulfonic acid, crystals formed at once. After 2 more days at 3°, the crystals were filtered off,

[2] The natural isomer was used. The nomenclature is based on spatial configuration, not on optical behavior.

[3] The writer is indebted to Dr. Max Bergmann for generous samples of the salts used in the leucine and phenylalanine derivatives.

washed with a small volume of cold N HCl, and dried *in vacuo* over P_2O_5. The yield was 1.62 gm. of material containing 3.4 per cent N (calculated 3.5 per cent N). The composition did not change upon recrystallization and very little loss in weight resulted. The leucine isolated as the 2-bromotoluene-5-sulfonic derivative corresponded to 6.1 per cent of the virus.

. The experiment was repeated, except that the residue from the 50 ml. was dissolved in 5 ml. of 2 N HCl, made to a volume of 20 ml., and 2.6 gm. of the reagent added. The behavior of the solution upon being seeded with the known leucine derivative was identical with that in the first experiment. In this case, 2.338 gm. containing only 2.4 per cent N were obtained. Upon recrystallization from a small volume of 0.5 N HCl, 1.60 gm. of crystals containing 3.3 per cent N were obtained. Evidently the first crop contained some of the reagent. The identity of the precipitates was further established by means of solubility tests. They exhibited the same solubility as the *l*-leucine derivative prepared in the same manner and were not measurably soluble in N HCl saturated with the known derivative. Hence, the virus appears to contain at least 6.1 per cent leucine.

Colorimetric estimation (22) indicated the presence of 2.2 gm. of phenylalanine, corresponding to but 2.3 per cent of the protein. Apparently serious losses were encountered during the fractionation process. A 25 ml. portion of the fraction was evaporated to dryness under reduced pressure and taken up in 10 ml. of N HCl. To the hot solution, 0.42 gm. of the sodium salt of 2,5-dibromobenzenesulfonic acid was added. Crystals formed as the solution cooled. After standing at 0° for 2 days, they were filtered off, washed with a small volume of cold N HCl, and dried *in vacuo* over P_2O_5. 0.4244 gm., containing 2.8 per cent N, was obtained. A weighed portion was recrystallized. The recrystallized salt contained 2.7 per cent N (calculated 2.9 per cent), exhibited the same solubility in N HCl as a sample of a phenylalanine derivative, and was not measurably soluble in a saturated solution of the latter. The amount isolated corresponded to 1.8 gm. of phenylalanine in the entire sample.

Another portion, amounting to 100 ml., was treated in an identical manner, except that the amino acids were taken up in 20 ml. of N HCl and 0.83 gm. of the reagent was added. The precipitate weighed 1.039 gm. and contained 2.7 per cent N. The crystalline salt contained 2.9 per cent N and was obtained in an amount corresponding to a total of 1.8 gm. of phenylalanine in the fraction.

SUMMARY

Of the amino acids previously reported in tobacco mosaic virus protein, arginine, phenylalanine, tyrosine, and proline were isolated from hydrolysates of the virus. As expected, the amounts actually isolated were

less than those previously found. In addition, the presence of at least 5.3 per cent glutamic acid, 2.6 per cent aspartic acid, 6.1 per cent leucine, 3.9 per cent valine, and 2.4 per cent alanine in the virus was established by isolation procedures. The present work brings the total of the known constituents of tobacco mosaic virus to about 68 per cent.

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PHOTOELECTRIC DETERMINATION OF *dl-α*-TOCOPHEROL IN SERUM*

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The following methods have been proposed for the determination of tocopherol in biological material: the colorimetric α,α'-bipyridine method, the potentiometric gold chloride titration, and the red color reaction with alcoholic nitric acid. During the study of vitamin E in the serum of normals and of patients suspected of abnormal tocopherol metabolism, we found the need for a method for analysis of tocopherol in high dilutions.

The bipyridine method has been developed by Emmerie and Engel for the assay of tocopherol concentrates (1). They also used it for the study of the tocopherol level in the serum of tocopherol-deficient and tocopherol-treated rats (2). We have adapted their method for the analysis of human serum by using a photoelectric colorimeter, since the pink color of the iron-bipyridine complex is not very suitable for visual comparison. Our method follows in general the principle laid down by Emmerie and Engel. Because of the difficulty of obtaining their original articles it seems appropriate to summarize their procedure. They introduce formaldehyde and 95 per cent ethyl alcohol into the alkaline digestion and follow this procedure by a triple ether extraction. The ether of the washed and dried extract is replaced by benzene and this solution passed through a floridin column for the removal of carotenoids. The colorless filtrate is brought to reaction with the ferric chloride-α,α'-bipyridine reagent, and the color is read in a Pulfrich photometer.

We have found the following measures necessary to achieve satisfactory results: Floridin, 30/60 mesh, activated at 480° is suitable for the adsorption of carotene and vitamin A. Interference by traces of phosphatides is eliminated by the use of cadmium sulfate, as we found sulfuric acid alone insufficient in the case of serum. It is necessary to perform all evaporation and filtration under nitrogen. This holds especially for adsorption on the

floridin column, which must be filled with nitrogen before operation. It, seems that otherwise the oxidation products formed from carotene are not completely adsorbed by the column.

All procedures are carried out in amber-colored glassware for protection against light. If this is not available, glassware may be wrapped in orange (Tango) cellophane. In addition, all colorimetry must be carried out in a dark room, since light not only affects the vitamin E itself but causes a false color reaction in the blank.

Method

10 ml. of serum from a fasting subject are treated in a 250 ml. separatory funnel with 5 ml. of 0.2 N aqueous potassium hydroxide solution; 15 ml. of 37 per cent formaldehyde, which should be neutral toward phenolphthalein, and 15 ml. of 95 per cent alcohol are added. After complete precipitation of the proteins by thorough shaking, 50 ml. of peroxide-free ether are added.[1] The ethereal layer, which contains vitamin E as well as vitamin A and carotenoids, is separated. The aqueous layer is extracted twice more with 50 ml. portions of ether, after addition of 10 ml. of ethyl alcohol. The combined ether extracts are washed with 25 ml. of 2 per cent potassium hydroxide.

We have found that the subsequent treatment of the ether layer twice with 15 ml. of 1 per cent sulfuric acid results in turbidity, presumably due to certain phosphatides. Addition of 1 per cent cadmium sulfate to the 1 per cent sulfuric acid, while not preventing the appearance of turbidity, subsequently causes its disappearance when the ether extract is shaken three times with about 25 ml. of 0.5 per cent sodium sulfate solution. The ether extract thus washed acid-free is dried over anhydrous sodium sulfate for 1 hour. The dried solution is filtered in a nitrogen atmosphere through a layer of anhydrous sodium sulfate into a filter flask. The latter is immersed in warm water, and the ether is evaporated, the temperature of the bath being gradually raised to 55°.

Before complete dryness is reached, one adds 10 ml. from a supply of dry benzene, which has been freshly distilled and kept over sodium wire. The

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[1] We find it necessary to test the ether not only for the absence of peroxide, but also for the absence of any coloring matter whatsoever. The testing was done by complete evaporation in a filter flask of 400 ml. of ether, the total amount used for the determination; 5 ml. of benzene, added to the flask after evaporation, should not assume any color. Furthermore, when the ferric bipyridine reagent is added to this benzene solution, no more than a trace of pink color should develop; correction for this small error is included in the "distilled water" blank mentioned under "Determination of samples." Most of the present work was carried out with Baker's Analyzed absolute ether, c.p., kept over sodium wire.

benzene is evaporated, and the addition and evaporation of more benzene in small portions is repeated until the residue forms a clear solution with 5 ml. of benzene; a total of about 25 ml. of benzene is usually required to reach this point. The clear yellow benzene solution is now passed under nitrogen through a column of floridin earth of 12 mm. diameter and 30 mm. height, which retains vitamin A and carotenoids. These substances cause the floridin layer to assume a dark greenish blue color, but it may be used over again for about five successive analyses. The column is washed with 25 ml. of dry benzene, and the colorless filtrate is again evaporated under nitrogen to dryness. To remove traces of moisture, another 10 ml. of dry benzene are added, and evaporation to dryness is repeated. The pure tocopherol is taken up in 2.5 ml. of dry benzene and is now ready for development of the color reaction.

Preparation of Standards

A curve is constructed from the galvanometer readings of standard dl-α-tocopherol solutions in a Klett-Summerson photoelectric colorimeter.

In order to obtain the same mixture of solvents as in the sample itself, namely 1 part of benzene to 9 parts of absolute ethanol, we first place 2.5 ml. of benzene in each of a set of 25 ml. volumetric flasks. We then add 0.1, 0.2...3.0, 3.5, 4.0 ml. of a solution containing 10 mg. of tocopherol in 100 ml. of absolute ethyl alcohol. In each of these flasks are then placed 1 ml. of a 0.5 per cent solution of the α , α'-bipyridine reagent and 1 ml. of 0.2 per cent ferric chloride, both in absolute ethyl alcohol. All are then made up to the 25 ml. mark with absolute ethyl alcohol. After they have stood for 10 to 15 minutes, the extinction is determined with a green filter, Klett No. 52, which transmits light from 485 to 550 mμ. A blank is prepared along with the standards, containing 2.5 ml. of benzene and the reagents, and made up to 25 ml. as above. The instrument is now adjusted to the zero reading with the blank solution, and the various standard dilutions are read.

The following are mean values of numerous series carried out on different days.

dl-α-Tocopherol in 25 ml. final reaction mixture, γ	10	20	50	100	150	200	250	300	350	400
Galvanometer reading, logarithmic scale	9	18	42	84	126	168	210	252	294	336

The curve constructed from these values is a straight line in the given range (3).

Determination of Samples

2.5 ml. obtained as the final benzene extract are treated with the reagents identically as the standards, made up to 25 ml., and read after zero adjustment of the colorimeter with a blank of the reagents. This adjustment does not take into account a small amount of color produced by contaminants in the ether used for extraction. We find it necessary periodically

TABLE I

Duplicate Determinations of Tocopherol Content of Human Serum

Sample No.	Serum	Tocopherol found		Average
	ml.	γ	*mg. per cent*	*mg. per cent*
1-a	5	24	0.48	0.50
1-b	5	26	0.52	
2-a	5	96	1.92	1.92
2-b	5	96	1.92	
3-a	6	93	1.55	1.59
3-b	6	98	1.63	
4-a	7	60	0.86	0.89
4-b	7	65	0.93	
5-a	10	86	0.86	0.90
5-b	10	94	0.94	

TABLE II

Recovery of Tocopherol Added to Serum Samples

	Serum	Tocopherol			Recovery $100 (F - S)/A$
		Serum, S	Added, A	Found, F	
	ml.	γ	γ	γ	*per cent*
Man 1.........	6	35	48	72	77
" 2...........	9	96	48	139	90
" 3	8	50	84	132	97
" 4.........	4	42	200	225	91.5
Dog........................	6	74	200	304	115
Man 5.....	5	40	282	292	89
Horse serum (preserved)	8	18	282	248	81.5

to go through the entire procedure with distilled water instead of the serum sample. The resulting correction amounts to eight to ten scale divisions, and this amount has to be subtracted from the actual reading. The corrected reading is then interpolated from the curve, and the tocopherol content in 100 ml. is obtained by multiplication by 10, when 10 ml. of serum have been extracted. Generally, for x ml. of serum, the value on the curve must be multiplied by $100/x$.

Table I illustrates the reproducibility of results in determinations of duplicate samples.

The level of tocopherol in normal human serum varies from 0.6 to 1.4 mg. in 100 ml. These values are considerably raised upon oral administration of tocopherol (4). The low value for horse serum given in Table II is presumably caused by tocopherol destruction during storage. Emmerie and Engel (2) give values from 0.3 to 1.1 mg. per cent for rats receiving vitamin E.

It should be realized that the present method is not so specific as to afford direct identification of the ferric ion-reducing substance in the ether extract as tocopherol. The analytical recovery of tocopherol added to serum extract is, of course, irrelevant for this question. However, the assumption that the basal color reaction is due to tocopherol is corroborated by the diminution of this color in tocopherol-deficient rats, as observed by Emmerie and Engel (2), and also by the immediate and marked increase of the color reaction upon oral administration of tocopherol (4).

Recovery of Known Amounts of Tocopherol Added to Serum Extracts

A sample of serum was divided into two equal portions. To one portion a known amount of tocopherol dissolved in absolute ethyl alcohol was added. Parallel analyses of the two portions were carried out as above. The results are recorded in Table II.

SUMMARY

The amount of *dl-α*-tocopherol in samples of human serum of 10 ml. or less may be determined in the photoelectric colorimeter by a method consisting of an adaptation of the α,α'-bipyridine-ferric chloride method of Emmerie and Engel. A number of details of procedure are pointed out.

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THE CRITICAL PEPTIZATION TEMPERATURES OF ZEIN IN CONCENTRATED ETHYL ALCOHOL

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Zein is commonly assumed to be insoluble in ethyl alcohol containing less than 5 per cent of water and has been so reported by Osborne (1) and Swallen (2). Dill (3) has determined its critical peptization temperatures in a wide range of aqueous alcoholic mixtures but has confined his data to solutions containing less than 95 per cent alcohol by volume.

During the course of an extended inquiry into the characteristics of zein dispersions in various single and mixed solvents, the writers have extended Dill's data to mixtures containing less than 5 per cent of water and have found that zein is dispersible in concentrated aqueous alcohol as well as
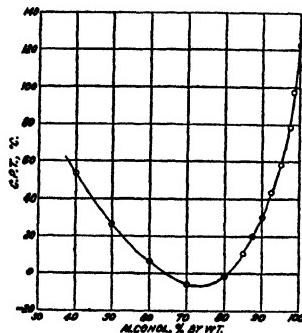
FIG. 1. Critical peptization temperature curve for zein in aqueous ethyl alcohol

in absolute alcohol at temperatures above the normal boiling point of the solvent.

However, unlike the solubility behavior of zein in most aqueous ethyl alcohol mixtures in which the critical peptization temperature and the cloud point lie in very close proximity, the temperatures of peptization in these concentrated and absolute alcoholic solutions are indefinite, and may be as much as 20° higher than their cloud points. For this reason, the values on Fig. 1 for 92.5 per cent to absolute alcohol are actually cloud points rather than critical peptization temperatures.

The data for the cloud points in aqueous alcohol solutions containing

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less than 95 per cent alcohol are in essential agreement with the results of Dill (3).

The absolute ethyl alcohol was prepared by refluxing over magnesium ethylate according to the method of Lund and Bjerrum (4). The zein, which was from a special lot used as a reference standard by the Corn Products Refining Company and by Prolamine Products, Inc., was dried in a vacuum over phosphorus pentoxide at 110° for 24 hours. A Carius tube was baked dry under a vacuum at 200°, and charged with 0.5 gm. of the dried zein, followed by 10 ml. of the alcohol distilled directly into the Carius tube in a closed system from the magnesium ethylate dehydrating agent. The tube was sealed in such a manner that its contents were not at any time exposed to the atmosphere. It was then immersed in an oil bath and heated until the zein had completely dispersed, after which the bath was allowed to cool slowly and the cloud point noted.

In a similar manner, the critical peptization temperatures of dried zein were determined in a series of ethyl alcohol mixtures containing up to 60 per cent of water. Beyond this concentration of water the zein tends to denature before reaching the temperature presumably required to effect its dispersion.

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THE SOLUBILITY OF CALCIUM PHOSPHATE

I. THE EFFECT OF pH AND OF AMOUNT OF SOLID PHASE

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In 1937, Logan and Taylor (1) came to the conclusion "that the bone salts cannot precipitate spontaneously from the bone plasma unless the ion product is increased. Once formed, the bone salts cannot dissolve unless the concentrations of the ions composing them are decreased below the concentrations found in the blood plasma." This conclusion was based upon some observations which seemed to Logan and Taylor to prove that the apparent solubility product, $[Ca^{++}]^3[PO_4^{\equiv}]^2$, depended on the amount of the solid phase, being greatest with small amounts and decreasing with larger amounts. The solid phases they employed were precipitated calcium phosphate and powdered bone. Such a change of ion product, at equilibrium, with the amount of solid phase is, of course, quite remarkable and, in order to explain their results, Logan and Taylor resorted to as remarkable an explanation. We will discuss their experiments and their explanation later.

A recent paper by Klement and Weber (2) leads to a quite different conclusion from that of Logan and Taylor. Klement and Weber prepared a hydroxyapatite by mixing dilute solutions of $Ca[OH]_2$ and of H_3PO_4 in the equivalent proportions. Their preparation was, as they stated, assuredly free of foreign ions, but there is no evidence that it was a single chemical individual. No such claim was made for it. The precipitate was filtered and dried. Weighed quantities of from 100 to 1200 mg. were added to 250 ml. of conductivity water in paraffined flasks and equilibrated for 24 hours at 25°. The amounts of Ca dissolved varied from 3.6 to 11.0 mg. per liter and those of P from 8.6 to 32.2 mg., increasing regularly with the amount of solid phase. Upon the basis of this experiment, Klement and Weber deny the significance of the solubility product $[Ca^{++}]^3 [PO_4^{\equiv}]^2$.

However, it may well be objected that 24 hours may not have been sufficient for the attainment of equilibrium.

We have been engaged in a study of the influence of various substances upon the solubility of calcium phosphate. In the course of this work we found that this solubility, whether it be expressed as $[Ca^{++}]^3 [PO_4^{\equiv}]^2$ or, as we prefer, as $[Ca^{++}]^3 [HPO_4^-]^2/[H^+]^2$, is not constant,[1] but varies with the

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[1] In the present instance, calculations of the product $[Ca^{++}]^3 [PO_4^{\equiv}]^2$ are impossible because no good values for the third dissociation constant of H_3PO_4 at $\mu = 0.2$

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amount of the solid phase, not, however, in the manner reported by Logan and Taylor, but in precisely the opposite fashion; *viz.*, the solubility increases with the amount of solid phase.

We also found that the apparent solubility product varies with the pH, increasing as the latter increases. This effect is not appreciable with small amounts of solid phase, but becomes considerable as the latter increases.

EXPERIMENTAL

In order to enable determinations of calcium and phosphorus of the order of 10^{-4} M to be made in the presence of salts in a concentration of 0.2 M, the Ca and P were added in the form of a suspension of known content and the amount remaining undissolved was determined.

A suspension of calcium phosphate was prepared by mixing 2.5 liters of 0.04 M $Ca(NO_3)_2$, 2.5 liters of 0.04 M Na_2HPO_4, and 200 ml. of 0.1 N NaOH. After the mixture had stood, with frequent stirrings, for a week, the precipitate was washed by decantation until a test of a portion dissolved in dilute acetic acid gave no precipitate with nitron, and for several times thereafter. The precipitate was then kept in suspension for at least a year before use. As required, more dilute suspensions were prepared and analyzed. The figures in Table I show slightly varying values for the ratio of Ca:P. These are for different dilutions of the stock suspension, each of which was separately analyzed at widely separated intervals with different standard solutions.

Measured volumes of these suspensions were added to 1 or 2 liter volumetric flasks containing H_2O, KCl to give a final concentration of 0.2 M, 10 ml. of 0.04 per cent chlorophenol red or phenol red solution per liter,[2] and small quantities of 0.1 N HCl or 0.1 N NaOH. After the solutions were diluted to the mark, the stoppered flasks were allowed to stand for the number of days indicated in Table I; they were shaken once almost every day. The precipitate settled very slowly, separation being quite incomplete even after 6 or 8 or even 24 hours.

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appear to be available. Other reasons for preferring $[Ca^{++}]^3 [HPO_4^{-}]^2/[H^+]^2$ are presented in Paper II. The values for $-\log [Ca^{++}]^3 [HPO_4^{-}]^2/[H^+]^2$ given in Table I can be converted into values for $pK'_{sp} [Ca^{++}]^3 [PO_4^{-}]^2$ by adding $2pK'_3$ whenever values for the latter become available. However, the use of neither product is theoretically valid at pH > 6. The solid phase, at equilibrium, does not have the composition indicated by $Ca_3(PO_4)_2$. The use of the third power of the calcium concentration and the second power of the phosphate concentration in this paper is due only to the fact that such a product has been used by others, particularly by Logan and Taylor.

[2] These indicators were added in order that the pH of these mixtures, which were to serve as controls, should be nearly the same as those of other mixtures containing other substances.

When analyses were to be begun, the mixtures were again shaken thoroughly and the pH was determined with the Coleman glass electrode. The cell was flushed with the mixture until no change in pH was observed. Four or five rinsings sufficed. Measured portions of the mixture were then filtered, with suction, through sintered glass filters covered with a thin

TABLE I

Effect of pH and of Amount of Solid Phase on Solubility of Calcium Phosphate in 0.2 M KCl

Experiment No.	Days	pH	Added		Dissolved		$-\text{Log} \frac{[Ca^{++}]^3 \times [HPO_4^-]^3}{[H^+]^2}$
			Ca	P	Ca	P	
			$M \times 10^6$	$M \times 10^6$	$M \times 10^6$	$M \times 10^6$	
1	29	6.50	16	10	7	4	14.43
2	17	6.98	16	10	3	2	14.49
3	16	6.90	32	20	15	11	11.14
4	9	6.92	32	20	13	7	11.14
5	13	6.92	32	20	14	7	11.47
6	24	7.30	31.5	20	16	8.5	10.19
7	13	6.88	63	40	38	24	9.31
8	40	6.30	80	50	56	35	10.38
9	22	6.50	80	50	53	36	9.61
10	21	6.70	80	50	55	33	9.10
11	20	6.90	80	50	46	27	8.90
12	7	6.95	80	50	34	20	9.41
13	10	7.28	80	50	34	18	8.61
14	4	8.90	80	50	18	8	6.64
15	8	7.40	126	80	61	37	7.87
16	29	5.90	240	150	206 (195)	128 (128)	9.07
17	21	6.05	480	300	441 (440)	272 (276)	6.82
18	14	6.30	480	300	394	248 (246)	6.14
19	7	5.80	640	400	579 (588)	365 (375)	7.14
20	13	6.20	640	400	496	316 (329)	5.97

The figures in parentheses were obtained by direct analysis of the filtrates. In these instances, the averages of these values and those obtained by subtracting undissolved Ca (or P) from total added Ca (or P) have been used for calculating the figures in the last column.

layer of infusorial earth. This was prepared by washing commercial acid-washed infusorial earth with concentrated HCl and then with H_2O until free of acid. As much as 400 ml. was taken for the determination of Ca and 75 ml. for the determination of P.

The precipitates were not washed,[3] but were sucked dry and were then

[3] If the amounts of Ca and of P contained in the liquid adhering to the precipitate and filter were appreciable, their effects should have been most marked in those

dissolved in approximately 0.02 N HCl, which was allowed to run through by gravity. In these filtrates, evaporated if necessary, phosphorus was then determined by the method of Fiske and Subbarow (3) and calcium by precipitation as the oxalate, by centrifuging, washing, and titrating with KMnO$_4$.

In a number of instances, the original filtrate contained enough Ca and P to permit of direct determination. Quite good agreement with that calculated by difference was obtained. Repeatedly, the filtrates obtained in two or three determinations were combined and passed through other filters. The latter were then extracted in the usual manner. No trace of Ca or of P was obtained, indicating that the previous removal of Ca and P was due to filtration and not to adsorption.

For the calculation of $-\log [Ca^{++}]^3 [HPO_4^-]^2/[H^+]^2$ we used the value pK$'_2$ = 6.85, calculated from the formula of Cohn (4). Those who prefer to use $[Ca^{++}]^3 [PO_4^-]^2$ may add 2pK$'_3$.

DISCUSSION

In all but one of our experiments, "equilibration" was continued for at least 7 days. As may be seen from the results of Experiments 11 and 12, or from those of Experiments 3, 4, and 5, prolongation up to 20 days increased the solubility comparatively little, if at all (Table I).

The increase in the amount of calcium and phosphate in solution with the increase in the amount of solid phase is not surprising. It has long been known (see Eisenberger, Lehrman, and Turner (5) for a review of the literature) that calcium phosphate, in contact with aqueous solutions, rarely, if ever, has the composition Ca$_3$(PO$_4$)$_2$. Usually, at pH 6.0 or more, it contains an excess of calcium. If it is not a pure substance, but a mixture, increasing the amount added to a liquid may increase the amount dissolved up to a certain maximum. Apparently, that maximum, at pH 6.3, was approached in Experiment 18, for a 25 per cent increase in the amount of solid phase added (Experiment 20) produced a comparatively small change in the ion product.

It is remarkable that, in spite of all that has appeared in biological literature regarding the solubility product $[Ca^{++}]^3 [PO_4^-]^2$, the validity of the use of this formulation has never been tested save over a very limited range of pH. With one exception, to be mentioned presently, all determinations have been made between pH 7.10 and 7.60. In spite of that,

experiments in which the ratio of dissolved to undissolved material was greatest. That was the case in Experiment 19 in which the ratio was 10:1. Nevertheless, the value for dissolved material obtained by difference was within 1.6 per cent (Ca) or 2.7 per cent (P) of those obtained by direct analysis of the filtrate. It was, therefore, concluded that the correction for adherent liquid was negligible.

the variations even with large excess of solid phase are considerable—at least 10-fold (see the data of Sendroy and Hastings (6)).

The one exception to this restriction of measurements to a limited pH is that furnished by some observations of Holt, La Mer, and Chown (7). They approached equilibrium from supersaturation and determined the solubility of calcium phosphate in the presence of the precipitate in NaCl or KCl solution at pH 5.2 to 5.5 and in "serum salt" solutions at pH 6.9 to 7.4. At comparable ionic strengths the ion product was about 100 times as great in "serum salt" solutions as in KCl or NaCl solutions. Even if the ion product is recalculated by the method of Sendroy and Hastings and also after allowance is made for the presence of undissociated $CaHPO_4$, $CaCO_3$, and $CaHCO_3$ (8, 9), the difference remains nearly as great. It is interesting to observe, therefore, that our Experiments 8 and 13 show a difference of 1.77 in the logarithms of the ion products between pH 6.30 and 7.28 and that other experiments show smaller variations over smaller variations in pH.[4]

The effect of pH on the apparent solubility product cannot be due to an error in the value of pK'_2, for so extreme a change of the latter from 6.85 to 6.50 still leaves a difference in the value of $-\log[Ca^{++}]^3 [HPO_4^=]^2/[H^+]^2$ amounting to 0.62 between Experiments 8 and 13, to 0.45 between Experiments 17 and 18, and to 1.08 between Experiments 19 and 20.

There remain to be discussed the reasons for the results obtained by Logan and Taylor. We believe these to have been due to insufficient equilibration. They approached equilibrium from supersaturation and their sole test for completeness of equilibrium appears to consist of one experiment in which they brought together an unstated excess of Ca^{++} and Na_2HPO_4, centrifuged to remove the precipitate, added NaOH to adjust the pH for the acidification due to the precipitation, and repeated the process until no further obvious precipitation was produced. The three solutions were then shaken with a *small* quantity of the precipitate for 4 and 8 days. In their paper, Logan and Taylor did not report the actual concentrations nor the amount of solid phase added, but Dr. Logan kindly made the former of these available to me. They are given in Table II, together with the values for pH and for $-\log[Ca^{++}]^3 [PO_4^=]^2$ reported by Logan and Taylor in their paper. The amount of the solid phase added is not known but a comparison of the values for $-\log[Ca^{++}]^3 [PO_4^=]^2$ with those reported by Logan and Taylor in other experiments (their Table I) would indicate that it was less than 2.5 mg. and probably less than 1 mg. per liter. 1 mg. per liter is the equivalent of 0.01 mM of Ca and of 0.0065

[4] It is also possible that the differences in the calculated ion products in the two series of experiments of Holt, La Mer, and Chown were due to the formation of mixed complexes of Ca^{++}, HCO_3^-, and HPO_4^-.

MM of P.ʹ Actually, the difference between the concentrations observed at 4 and at 8 days far exceeds these quantities. In the second experiment, [P] is said to have changed from 4.60 to 5.40 mm, an *increase* of 0.8 mm, while the [Ca] *decreased* by 0.02 mm. These changes in the solution would make a very great difference in the composition of the solid phase. With even so large a quantity as 310 mg. of $Ca_8(PO_4)_2$, the ratio of Ca:P

TABLE II

Composition of Fluid after 4 and 8 Days (Experiments of Logan and Taylor)

4 days*			8 days*			Reported by Logan and Taylor (1)		
pH	Ca	P	pH	Ca	P	pH	4 days $-\log$ $[Ca^{++}]_3$ $\times [PO_4^{=}]_2$	8 days $-\log$ $[Ca^{++}]_3$ $\times [PO_4^{=}]_2$
	mM	*mM*	ʹ	*mM*	*mM*			
7.12	0.75	5.25	7.15	0.76	5.02	7.15	23.43	23.52
7.44	0.74	4.60	7.37	0.72	5.40	7.35	22.91	22.95
8.10	0.57	1.06	7.93	0.62	1.03	8.10	23.37	23.37

* Personal communication from Dr. Logan to the author.

TABLE III

*Data of Logan and Taylor**

Composition of the solution at the start, $CaCl_2$ 1.06 mm per liter, Na_2HPO_4 3.06 mm per liter.

Experiment No.	Solid added Bone	Calculated as $Ca_8(PO_4)_2$	Composition of solutions after equilibration			$p[(Ca^{++})_3 \times (PO_4^{=})_2]$	Total $Ca_8(PO_4)_2$ in solid phases
			pH	Ca	P		
	mg. per l.	*mg. per l.*		*mm per l.*	*mm per l.*		*mg. per l.*
61	1.3	0.74	7.43	1.056	3.06	22.77	0.74
62	5.0	2.87	7.38	0.929	2.98	23.09	15.0
63	25.0	14.3	7.34	0.619	2.79	23.74	57.0
64	49.0	28.1	7.29	0.467	2.66	24.28	89.0
65	100.0	58.0	7.22	0.365	2.66	24.80	124.0

* The first seven columns are taken from Logan and Taylor ((1) p. 299).

would change from 1.5 to 2.5. Such experiments cannot be accepted as evidence of "equilibrium." If this was not attained in these experiments, there can be no reason for believing that it was attained in the later experiments. The lower values for $[Ca^{++}]^3$ $[PO_4^{=}]^2$ with increasing amounts of solid phase are, obviously, only an indication of a more nearly complete approach to true equilibrium, which is only to be expected.

Logan and Taylor explain the lowered solubility with increases in solid phase as being due to adsorption of Ca^{++}, $PO_4^{=}$, and other ions upon the

true precipitate or upon the solid phase introduced. We may test this assumption by calculations, the results of which are summarized in Table III in which all but the last column is copied from Table II of Logan and Taylor. From the changes in [Ca] and [P] reported by them, we have calculated the amount of $Ca_3(PO_4)_2$ precipitated in each experiment. Adding this value to the amount originally present, we have the figures given in the last column. According to the hypothesis of Logan and Taylor, the 2.13 mg. of additional $Ca_3(PO_4)_2$ added in Experiment 62, as compared with Experiment 61, adsorbed 12 mg. of $Ca_3(PO_4)_2$. This cannot be "adsorption" as ordinarily understood. It must be crystal growth. It is also obvious from an examination of Table II that, *at the time of analysis*, the amount of solid phases present in Experiment 62 was greater than was originally added in Experiment 63. The same relation holds between Experiments 63 and 64 and between Experiments 64 and 65. It is, we believe, impossible to interpret the increases in the value of pK'_{sp} $Ca_3(PO_4)_2$ with increases in the amount of bone introduced except as evidence of continued supersaturation in Experiments 61 to 64.

We believe that the evidence that the results of Logan and Taylor were due to incomplete equilibration is overwhelming. We freely admit that our results may, similarly, be due to incomplete equilibration. A true equilibration can be demonstrated only if the same conditions are attained from both undersaturation and oversaturation. Quite evidently, this can be attained, in a reasonable length of time, only with considerable amounts of precipitate. With small amounts, equilibrium is, apparently, not to be attained in any feasible period, for a continuation of the experiment much beyond 8 days at 38° involves serious risk of reaction with the walls of the vessel. The results reported by Logan and Taylor and in the present paper can be reconciled if one accepts the view that the composition of the precipitate, approximately $Ca_3(PO_4)_2$, depends upon the composition of the liquid and that changes in this composition, involving a change from one insoluble substance into another, take place only very slowly. An apparently true equilibrium constant, pK'_{sp} = approximately 27 at 38°, can be attained with large amounts of precipitate because, with the large surface offered, equilibrium at the *surface* is more readily attained. It is this surface and not the bulk of the precipitate that is believed to be in equilibrium with the solution.

SUMMARY

The claim of Logan and Taylor (1) that "The ion product $[Ca^{++}]^3$ $[PO_4^=]^2$ increases as the amount of bone or tricalcium phosphate, equilibrated with solutions of their ions, decreases below 150 mg. per liter" has been examined. It is believed that the data presented by them are more in

accord with the view that the mixtures containing the smaller amounts of precipitate, were supersaturated. Other experiments are reported which indicate that the reverse relation obtains; *viz.*, that the ion product increases with the amount of solid phase. Attention is also directed to the apparent effect of increasing pH on the ion product. It is believed that the composition of the precipitate depends upon the composition of the liquid but that changes in the former are brought about only very slowly and incompletely and, probably, only at the surface, within any period of time thus far employed.

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THE SOLUBILITY OF CALCIUM PHOSPHATE

II. THE SOLUBILITY PRODUCT

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While it has been customary to regard calcium phosphate as $Ca_3(PO_4)_2$, analyses do not agree with this formula but show an excess of Ca, accompanied by OH^-, CO_3^-, or other anion. From their review of the literature, Eisenberger, Lehrman, and Turner (1) have concluded that "between dicalcium phosphate and lime, there exists, in the ternary system, a continuous series of solid solutions having an apatite lattice. It follows from this that tricalcium phosphate and hydroxyapatite do not exist in aqueous systems as unique, stoichiometric compounds." Nevertheless, some method of comparing solubilities under different conditions is needed and the solubility product $[Ca^{++}]^3 [PO_4^=]^2$ has been used for this purpose since its introduction by Holt, La Mer, and Chown (2). However, the values found are not very consistent, variations of more than 10-fold being, apparently, quite acceptable. This means more than a 2-fold error in the calcium concentration, or a 3-fold error in that of phosphate, or a 10-fold error in that of $[H^+]$, or a 1.4-fold error, in the same direction, in all. These seem quite outside the limits of error of the determinations and seem to indicate that certain unknown factors are involved.

Greenwald, Redish, and Kibrick (3) have presented evidence for the existence, in solution, of undissociated $CaHPO_4$. Such a substance could act as an acid and might, conceivably, form a calcium salt of the composition $Ca(CaPO_4)_2$. Obviously, this can no more be a "unique, stoichiometric compound" in an aqueous system than can $Ca_3(PO_4)_2$. However, it seemed that it might be worth while to devise a procedure for the calculation of the ion product $[Ca^{++}][CaPO_4^-]^2$ in the hope that the values might be more consistent than those for $[Ca^{++}]^3 [PO_4^-]^2$.

Let

$$K'_{diss.} = \frac{[Ca^{++}][HPO_4^-]}{[CaHPO_4]} \tag{1}$$

and

$$K'_4 = \frac{[H^+][CaPO_4^-]}{[CaHPO_4]} \tag{2}$$

If we divide Equation 2 by Equation 1,

$$\frac{K'_4}{K'_{diss.}} - K'_5 = \frac{[H^+]\,[CaPO_4^-]}{[Ca^{++}]\,[HPO_4^-]} \tag{3}$$

from which

$$[CaPO_4^-] = \frac{K'_5 \cdot [Ca^{++}]\,[HPO_4^-]}{[H^+]}$$

$$K'_{sp}Ca(CaPO_4)_2 = [Ca^{++}]\,[CaPO_4^-]^2$$

$$= \frac{[Ca^{++}]\,[K'_5]^2\,[Ca^{++}]^2\,[HPO_4^-]^2}{[H^+]^2}$$

Therefore[1]

$$K'_{sp}Ca(CaPO_4)_2 = \frac{K_{sp}Ca(CaPO_4)_2}{(K_5)^2} = \frac{[Ca^{++}]^3\,[HPO_4^-]^2}{[H^+]^2} \tag{4}$$

If

$$K'_5 = \frac{[H^+]\,[PO_4^-]}{[HPO_4^-]}, \quad \frac{[PO_4^-]}{K'_5} = \frac{[HPO_4^-]}{[H^+]}$$

Substituting in Equation 4, we have

$$K'_{sp}Ca(CaHPO_4)_2 = \frac{[Ca^{++}]^3\,[PO_4^-]^2}{[K'_5]^2}$$

or $pK'_{sp}Ca(CaPO_4)_2 = pK'_{sp}Ca_3(PO_4)_2 - 2pK'_5$.

It might expected, therefore, that $pK'_{sp}Ca(CaPO_4)_2$ should show no lesser variation, at constant ionic strength, than does $pK'_{sp}Ca_3(PO_4)_2$.

Such a consideration neglects the errors inherent in the determination of the third dissociation constant of phosphoric acid. This is not nearly as accurately known as are the first two constants. Thus, Bjerrum and Unmack (4) claim an accuracy of 0.01 to 0.02 for their values of pK_1 and of 0.01 for that of pK_2, but only 0.03 to 0.04 for that of pK'_3. The values for pK'_1 and pK'_2 used by Sendroy and Hastings (5), at 38° and $\mu = 0.152$, differ from those calculated by the formulae of Bjerrum and Unmack by only 0.034 and 0.072, respectively, but that for pK'_3 differs by 0.204. The use of the product $[Ca^{++}]^3\,[HPO_4^-]^2/[H^+]^2$ obviates the difficulty caused by the uncertainty in the value of pK_3. While it is true that the value of K'_4 is absolutely unknown, this constant does not enter into our final equation. This requires for the phosphate term of the ion product only the total P, the pH, and K'_2 of H_3PO_4, all of which can be determined with a high degree of accuracy.

[1] K' is employed to indicate that the constant is not the product of $[Ca^{++}]$ by $\{CaPO_4^-\}^2$, but this value divided by the unknown value, $(K'_5)^2$, which probably changes with the ionic strength.

Moreover, it seems rather unreasonable to use the product $[Ca^{++}]^3$ $[PO_4^{\equiv}]^2$ which has never been demonstrated to have even the slightest validity in a region in which the fraction of the total phosphate that is present as $PO_4^{\equiv}$ is at all considerable. Such use as has been made of this product has been in a region in which $[PO_4^{\equiv}]$ is only 1 part in 10^4 to 10^9, generally 10^5, of the total phosphate and in which $[PO_4^{\equiv}]$ is approximately 10^{-8}.

Finally, the product $[Ca^{++}]^3$ $[PO_4^{\equiv}]^2$ implies a reaction of the fifth order which is rather improbable. Moreover, as has just been pointed out, one constituent is present in a concentration of 10^{-8} M. As was shown by

TABLE I

Solubility of Calcium Phosphate in NaCl Solutions

Data of Holt, La Mer, and Chown, recalculated by Sendroy and Hastings ((5) p. 809).

Experiment No.	pH	$\sqrt{\mu}$	$Ca \times 10^3$	$P \times 10^3$	$pK_{sp}Ca_3(PO_4)_2$		CaHPO₄ in solution $\times 10^5$	$pK'_{sp}Ca(CaPO_4)_2$	
					Found	From curve		Found	Calculated*
1	5.21	0.076	1.96	3.71	31.22	31.2	1.6	6.28	6.12
2	5.27	0.099	1.98	3.63	30.82	30.8	1.7	6.00	5.97
3	5.33	0.147	2.00	3.58	30.19	30.1	2.1	5.64	5.64
4	5.46	0.263	2.07	3.54	28.81	28.8	2.1	4.82	4.89
5	5.51	0.364	2.12	3.54	28.03	27.9	2.0	4.35	4.23
6	5.52	0.364	2.22	3.59	27.93	27.9	2.1	4.24	4.23
7	5.53	0.512	3.11	4.04	26.26	27.0	2.8	3.31	3.26

* Calculated from $pK'_{sp} = 6.62 - 6.57 \sqrt{\mu}$.

Bassett (6) and by others (2, 7, 8), if the solubility product for $CaHPO_4$ is exceeded, the immediate precipitate appears to be $CaHPO_4$. Only later, and rather slowly, may it change to that approximating the composition $Ca_3(PO_4)_2$. This is only to be expected. The formation of $CaHPO_4$ is a reaction of the second order, with both reactants present in at least milli-molar concentration. As a sparingly soluble acid, precipitated $CaHPO_4$ will slowly react with Ca^{++} in solution to form $Ca(CaPO_4)_2$. $CaPO_4^-$ in solution will react rapidly with Ca^{++}, the reaction being of the third order. Precipitation will, therefore, be rapid, although final equilibrium may be long delayed.

Reactions of the second order may also occur between $CaPO_4^-$ and $Ca(OH)^+$ or $Ca(HCO_3)^+$ (9). The regular occurrence of Ca in excess of that required by the formula $Ca_3(PO_4)_2$ is thus only to be expected.

It is interesting to note that the values for $pK'_{sp}Ca(CaPO_4)_2$, plotted against $\sqrt{\mu}$, fall on a straight line. For this purpose, we have used the

data of Holt, La Mer, and Chown for carbonate-poor solutions (2), as re-calculated by Sendroy and Hastings (5).[2] From these, and the values for $-\log [Ca^{++}][HPO_4^-]/[CaHPO_4]$ given by Greenwald, Redish, and Kibrick (3),[3] we calculated the value of $[CaHPO_4]$, and, after correcting total Ca and total P for this small quantity, the values for pK'_{sp} $-\log[Ca]^3 [HPO_4^-]^2$ /$[H^+]^2$ (Table I). Plotted against $\sqrt{\mu}$, they fall close to the straight line $pK'_{sp} = 6.62 - 6.57 \sqrt{\mu}$.

The significance of this equation is not clear. The relation between the ion product and $\sqrt{\mu}$ may be entirely fortuitous. However, the equation is useful in claculating the value of pK'_{sp} at 38° at any value of $\sqrt{\mu}$ from 0.076 to 0.512.

SUMMARY

It is proposed to regard tricalcium phosphate as the calcium salt of the acid $HCaPO_4$. It is believed that this makes more reasonable the rapidity of precipitation, the slowness with which equilibrium is attained, and the increasing content of Ca^{++} and OH^- or CO_3^- in the precipitate as the pH is increased. The use of the solubility product $[Ca^{++}][CaPO_4^-]^2 = [Ca^{++}]^3$ $[HPO_4^-]^2(K_5)^2/[H^+]^2$ obviates the need for the use of the third apparent dissociation constant of phosphoric acid, whose value is not known as accurately as is that of the second. Data in the literature lead to the equation, pK'_{sp} at 38° $= -\log [Ca^{++}]^3 [HPO_4^-]^2/[H^+]^2 = 6.62 - 6.57 \sqrt{\mu}$.

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[2] As it happens, these are the only determinations at pH < 6 in the literature and, therefore, the only ones in which the precipitate may be assumed to have the composition $Ca_3(PO_4)_2$. Moreover, the amount of solid phase was sufficiently great for "equilibrium" to be assumed. See Paper I for a discussion of the effect of the amount of solid phase.

[3] This involved a certain error, for pK_{diss}. was determined at about 20°, whereas the experiments of Sendroy and Hastings were made at 38°. However, the amount of $CaHPO_4$ was so small that a slight change in the value of K'_{diss}. could make no appreciable difference.

METABOLISM OF THE STEROID HORMONES

III. THE ISOLATION OF PREGNANDIOL-3(α),20(α) FROM THE URINE OF PREGNANT CHIMPANZEES*

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The gravimetric method of Venning (2) for the determination of sodium pregnandiol glucuronidate in human urine stimulated wide interest in this compound, which has been adequately shown to be a urinary metabolite of progesterone. The occurrence of sodium pregnandiol glucuronidate in the urine of women during the luteal phase of the menstrual cycle as well as during pregnancy is now well established. The pregnandiol complex has been found also in the urine of women with adrenal carcinomata (3, 4) and adrenal cortex hyperplasia (3), in the urine of both men (5, 6) and women (7–10) following the administration of progesterone and of sodium pregnandiol glucuronidate, and in the urine of men to whom desoxycorticosterone acetate was administered (11).

To the present time, however, the glucuronide of pregnandiol has not been found in the urine of any species other than man. Westphal and Buxton (12) failed to isolate the complex from the urine of normal and pregnant rabbits, normal and pregnant cats, and from the urine of monkeys injected with progesterone. Strickler, Walton, and Wilson (13) were unable to detect sodium pregnandiol glucuronidate in the urine of bulls. Likewise, Elder (14) could find no sodium pregnandiol glucuronidate in 24 hour specimens of urine from pregnant chimpanzees. In this laboratory, we have been unable to find sodium pregnandiol glucuronidate in the urine of guinea pigs to which progesterone and sodium pregnandiol glucuronidate were administered.[1]

Attempts to isolate unconjugated pregnandiol from the urine of man and other animals have met with success. Pregnandiol-3(α),20(α) and

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[1] Unpublished observations.

isomeric diols have been isolated from the urine of pregnant women (15–17), cows (18), and mares (19, 20), from the urine of bulls (21), and from the urine of women showing the adrenogenital syndrome (22). Heard, Bauld, and Hoffman (23) have reported, in a preliminary communication, the isolation of pregnandiol-3(α),20(α) from the urine of rabbits following the simultaneous administration of progesterone and α-estradiol or estrone. On the other hand, Marker and Hartman (24) reported that they were unable to isolate pregnandiol or any of its isomers from the urine of pregnant *rhesus* monkeys or from the urine of *rhesus* monkeys to which progesterone was administered.

In view of the close phylogenetic relationship of the chimpanzee to man and the apparent similarity of the reproductive physiology of these two forms, it seemed of considerable interest and importance to reexamine the urine of pregnant chimpanzees for the presence of pregnandiol. The fact that Elder (14) was unable to isolate pregnandiol in the form of its glucuronidate by the Venning procedure does not exclude the possibility that pregnandiol is actually excreted by the pregnant chimpanzee. First, the quantity of pregnandiol, if it is excreted as the glucuronide, may be too small to be isolated by the Venning procedure from 24 hour specimens. Second, pregnandiol may, in fact, be excreted in a form other than the glucuronide and thus be overlooked. It seemed advisable, therefore, to attempt the isolation of unconjugated pregnandiol or other metabolic products of progesterone. The results of such a study form the subject of the present communication.

EXPERIMENTAL

The urine from two healthy, pregnant chimpanzees, Wendy and Josie, was used. Urine was collected from Wendy from the 160th to the 171st day of pregnancy and from Josie from the 129th to the 140th day. All the specimens, representing 24 animal days, were pooled and amounted to 61 liters. 10 cc. of concentrated hydrochloric acid were added to each 100 cc. of urine at the time of shipment from Orange Park, Florida, to New Haven, where chemical examination of the urine was undertaken. Hydrolysis and extraction with benzene were carried out simultaneously by a modification of the method of Dingemanse, Borchardt, and Laqueur (25) described in Paper II of this series (26). The total compounds soluble in benzene were dissolved in ether and extracted with 10 per cent aqueous sodium hydroxide. The alkaline solution was reextracted with ether, which was added to the alkali-insoluble (neutral) fraction. The neutral fraction was washed with water and evaporated. Traces of water were removed from the residue by distilling absolute ethanol from it under reduced pressure.

After desiccation over phosphorus pentoxide, the neutral compounds were dissolved in 20 cc. of pyridine and 10 gm. of succinic anhydride were added. The mixture was heated on the steam bath for 90 minutes. The reaction mixture was rapidly cooled and partitioned between water and ether. The ether phase was separated and washed repeatedly with 10 per cent hydrochloric acid to remove the pyridine. The succinic half esters were extracted from the ether solution with 20 per cent aqueous sodium carbonate. The ether phase, containing the non-carbinols, was washed with water and evaporated to dryness.

The aqueous sodium carbonate solution, containing the succinic half esters, was acidified with 16 per cent sulfuric acid and extracted with ether. After evaporation of the ether, the residue was dissolved in 25 cc. of 20 per cent ethanolic (70 per cent) potassium hydroxide. This solution was boiled under a reflux condenser on the steam bath for 1 hour, diluted with cold water, and extracted with ether. The ether solution was then washed with water and evaporated. The residue, containing the carbinols, was dried by distilling absolute ethanol from it under reduced pressure.

In order to insure the complete separation of carbinols from non-carbinols, these fractions were again treated with succinic anhydride in pyridine and the respective fractions combined.

The carbinols were further separated into ketonic and non-ketonic fractions by means of the Girard-Sandulesco ketone reagent, trimethyl-acethydrazide ammonium chloride (27).

The non-ketonic carbinols were dissolved in 20 cc. of 90 per cent ethanol. To this solution were added 400 mg. of digitonin dissolved in 20 cc. of hot 90 per cent ethanol. After the mixture was allowed to stand 24 hours in the cold, a small quantity of digitonide was separated by filtration. The residue was washed with 90 per cent ethanol and with ether, the washings being added to the filtrate. The filtrate was concentrated to a syrup, dissolved in 25 cc. of pyridine, and heated on the steam bath for 1 hour. After the solution was cooled, 100 cc. of ether were added and the insoluble digitonin filtered off. The residue was washed with ether and the washings added to the filtrate. The filtrate was washed repeatedly with 10 per cent hydrochloric acid to remove the pyridine, with saturated sodium bicarbonate solution, and with water. The ether was then evaporated and the residue dried by boiling absolute ethanol from it under reduced pressure.

The non-ketonic, digitonin-non-precipitable carbinols were desiccated over phosphorus pentoxide and dissolved in 35 cc. of carbon tetrachloride. A portion of the material remained in suspension and was separated. Upon recrystallization twice from absolute ethanol, this material, insoluble

in carbon tetrachloride, weighed 13.3 mg. and melted at 234-236°.[2] When mixed with a sample of authentic pregnandiol-3(α),20(α) (m.p. 234-236°), the melting point was 234-236°. An additional 6.2 mg. of pregnandiol-3(α),20(α) (m.p. 231-233°) were obtained by dilution of the combined mother liquors.

The final mother liquor was evaporated and the residue dried over phosphorus pentoxide. The residue was then resuspended in carbon tetrachloride and combined with the non-ketonic, carbinolic fraction from which it was separated. The entire fraction was adsorbed on a column of activated alumina[3] (10 × 120 mm.).[4] The material was selectively eluted by passing through the column 50 cc. quantities of carbon tetrachloride containing progressively greater concentrations of absolute ethanol. Elution of crystalline material was effected by carbon tetrachloride containing 3 per cent of absolute ethanol. The residues from these eluates were combined and recrystallized twice from dilute ethanol. 28.8 mg. of crystals, m.p. 235-237°, were obtained. When mixed with authentic pregnandiol-3(α),20(α) (m.p. 234-236°), the melting point was 234-237°. The acetate melted at 174-176° and did not depress the melting point (176-178°) of a sample of authentic pregnandiol-3(α),20(α) diacetate.

DISCUSSION

The total quantity of pregnandiol-3(α),20(α) isolated from the urine of pregnant chimpanzees, 48.3 mg., represents the minimal amount present and is equivalent to 0.79 mg. per liter or an average daily excretion by each animal of 2 mg. These values are considerably lower than those reported for human pregnancy urine. They are similar in magnitude to those reported by Venning and Browne (7) and by Wilson, Randall, and Osterberg (28) for human non-pregnancy urine.

There are three possible sources of urinary pregnandiol in the pregnant chimpanzee, the corpus luteum, the placenta, and the adrenal cortex. Progesterone, which is known to be a precursor of pregnandiol in man and the rabbit, has been isolated from corpora lutea of sows (29) and from ox adrenals (30). Observations made by Smith and Kennard (31), which have been confirmed in this laboratory, demonstrate that human placentae contain progestational material, which may be progesterone. In the case of the adrenal cortex, pregnandiol might arise during the metabolism of steroids other than progesterone; e.g., desoxycorticosterone, which Reichstein and von Euw (32) isolated from the adrenal cortex (species?). At

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[2] All melting points are uncorrected.

[3] The activated alumina was procured from the Aluminum Ore Company, East St. Louis, Illinois; it is designated grade A, mesh 40.

[4] The insoluble material, which collected on the top of the adsorption column, was dissolved and carried through the column during elution.

the present time, the fate of desoxycorticosterone acetate in the chimpanzee is being investigated. In this connection, it is to be noted that Cuyler, Ashley, and Hamblen (11) have recovered sodium pregnandiol glucuronidate from the urine of men following the administration of desoxycorticosterone acetate. Further evidence that pregnandiol may arise during the metabolism of adrenal cortical compounds has been presented by Venning, Weil, and Browne (3) and by Salmon, Geist, and Salmon (4), who isolated sodium pregnandiol glucuronidate from the urine of women with adrenal carcinomata and adrenal cortex hyperplasia, and by Butler and Marrian (22), who isolated pregnandiol from the urine of women showing the adrenogenital syndrome.

SUMMARY

48.3 mg. of pregnandiol-3(α),20(α) were isolated from 61 liters of urine collected from two chimpanzees during the 5th and 6th months of pregnancy. This quantity is equivalent to 0.79 mg. per liter or an average daily excretion by each animal of 2 mg.

The reference sample of pregnandiol-3(α),20(α) was furnished by Dr. Russell E. Marker, to whom we express our thanks.

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OXIDATION, REDUCTION, AND SULFHYDRYL IN AUTOLYSIS

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Evidence has accumulated both for and against the idea that the control of the autolytic mechanism is in some way directly associated with oxidation-reduction levels in the tissue, and is mediated through sulfhydryl compounds such as glutathione and cysteine. Grassmann and Dyckerhoff first showed the activating effect of —SH and HCN on yeast proteases (1). Shortly after, Waldschmidt-Leitz *et al.* (2, 3) reported a similar activation of mammalian tissue proteases. They found an activator which increased as autolysis proceeded, and this was later identified as glutathione (4). Both groups of investigators indicated that the effect of —SH compounds was to increase the range of catheptic activity. Thus certain proteins not digested before the addition of —SH, were digested in its presence. Others were digested as well before activation as after. Kleinmann and his associates (5–7) showed activation of tumor proteinases toward gelatin, but failed to detect it when such native proteins as casein, egg albumin, organ proteins, and the proteins of the tissue from which the cathepsin was obtained were used. Abderhalden and his associates likewise were unable to detect activation toward the native and tissue proteins used, but found gelatin more rapidly digested in the presence of reduced glutathione (8). Mayr and Borger (9) showed that HCN increases the catheptic digestion of various proteins, including gelatin, serum albumin, edestin, and Witte's peptone. Reduced glutathione was more effective, while a combination of glutathione and HCN was the most effective activator found. On the other hand Bierich and Rosenbohm were unable to find activation by reduced glutathione in tumor tissues (10).

Voegtlin and Maver (11) showed that tissue autolysis was inhibited by oxygen, while the removal of oxygen by nitrogen gas permitted more extensive autolysis. They were able to correlate these effects with the loss or maintenance of sulfhydryl compounds reacting with nitroprusside. Voegtlin, Maver, and Johnson (12) showed that either protein hydrolysis or synthesis could be produced in an autolyzing tissue hash or by means of papain by properly adjusting the oxygen tension, and that the direction of the reaction was determined by the presence of —SH or —S—S— in the form of reduced or oxidized glutathione. Linderstrøm-Lang *et al.* (13–15) were unable to repeat this demonstration with papain and attributed the results previously reported to concentration of the digests by

the gas stream bubbled through them, or to cross-links formed on oxidation between the cysteine molecules of peptide chains which may produce compounds precipitated by trichloroacetic acid but which do not represent true protein synthesis. Maver and Voegtlin (16) have published further confirmation of the experiments on protein synthesis which indicates that the balance between substrate and products must be very accurately adjusted for this reaction to occur. Anson (17), working with a purified cathepsin, free from carboxypolypeptidase, has shown that the enzyme as prepared is already active toward hemoglobin and that no further activation results by adding cysteine. The enzyme does not digest gelatin. He also showed that polypeptidase is activated by cysteine. It becomes evident that gelatin, which has been so widely used as substrate material, may not indicate the presence of cathepsin, and that much of the reported catheptic activity is referable to the peptidases of the tissues. Maver has reported the preparation of a cathepsin which is activated by cysteine toward liver globulin (18).

More recently Fruton, Irving, and Bergmann (19) have presented evidence that cathepsin is a mixture of several different proteolytic enzyme factors which behave differently to certain pure synthetic substrate compounds and to activators such as cysteine. Just what the action of these enzyme factors is upon native proteins remains to be determined.

In view of the existing differences in the literature it has seemed to us profitable to reinvestigate the phenomena of activation and inhibition of the autolytic mechanism. In the present study we have concerned ourselves only with the changes which occur in whole liver digests. No foreign substrates have been added. Digestion, as measured by soluble nitrogen and the tyrosine color reaction, represents the action of the liver enzyme upon the liver proteins present. The results reported here are representative and typical examples and have been shown to be easily reproducible. We believe they are of significance in understanding the processes of atrophy, involution, and hypertrophy in the living tissue. Studies of the single purified enzymes of the system upon single pure substrate preparations will eventually be required to clarify the processes of autolysis. But in the meantime it seems desirable to know what happens in the natural substrate *milieu* of these enzymes as they are in the tissues themselves. Both lines of attack will supplement each other in clarifying the phenomena of changing protein mass in the living organism.

Effects of Varying Oxygen Tension on Liver Autolysis—Hog liver, obtained fresh from the abattoir, was ground to a fine hash and made up as a 20 per cent suspension in water with sufficient toluene to make 5 per cent of the final volume. Such a mixture was then homogenized in a Waring mixer, any inclusion of air bubbles during homogenization being avoided. The

creamy suspension was passed through a fine mesh sieve to remove shreds
of connective tissue. Such a suspension may then be accurately sampled
with an ordinary pipette. Digests were set up at a number of pH levels
by the addition of HCl or NaOH. Rapid streams of oxygen or nitrogen,
saturated with water and toluene vapor to prevent evaporation, were
passed through these digests in the cold for 15 minutes. The digests were
then placed in the thermostat at 38° and a slow stream of the gas bubbled
through them as long as the experiment lasted. Reflux condensers pre-
vented change in volume of the digests. Samples were removed from time
to time and precipitated with trichloroacetic acid whose final concentration
was 5 per cent or approximately 0.3 N. pH values were read daily on the
digests with a glass electrode, and adjustments made so that each digest
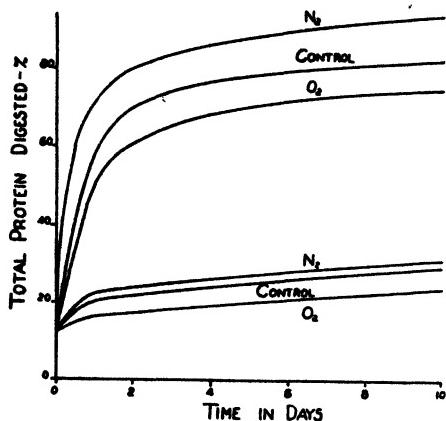
Fig. 1. The effect of aerobic and anaerobic conditions on the speed of autolysis at
pH 4 (upper set of curves) and pH 7 (lower curves).

was maintained at the initial H ion level throughout the experiment.
Digestion was determined by the soluble N_2 appearing in the trichloro
filtrate, and the increase of tyrosine, according to the Kjeldahl and Folin-
Ciocalteu techniques respectively. The usual series of digests was set and
maintained at pH 2, 3, 4, 5, 6, and 7.5. In several digests in this series
oxygen was bubbled through autolyzed mixtures which had been main-
tained anaerobic with a stream of nitrogen, in the hope of reproducing the
synthetic effects observed by Voegtlin. In no instance were we able to
discover evidence of protein synthesis under the conditions of our experi-
mentation. Typical results are shown in Fig. 1.

The points of significance appear to us to be the following.

1. The difference between anaerobic and aerobic autolysis, while not
large, is nevertheless significant and reproducible. Under anaerobic

conditions the digestion rate is highest and the extent of digestion in the 10 day period is greatest. Aerobic digestion is least. In this experiment we confirm the results reported by Voegtlin and Maver (11).

2. The effect of anaerobic or aerobic conditions is perceptible at all pH levels. It is greatest where digestion proceeds at the optimum pH, and least where digestion is always small, namely at pH 2, 2.5, 6, and 7.5.

3. The nitroprusside reaction disappears in 24 to 48 hours. It is most persistent in acid reaction and under anaerobic conditions when digestion is also optimum. This confirms the observation by Voegtlin and Maver (11).

4. The H ion concentration of the digest is the dominant factor in determining the speed and extent of autolysis. The oxidation level modifies this result up or down to a relatively small but significant extent.

5. We were unable to reverse the action from protein cleavage to synthesis by passing oxygen through digests previously subjected to anaerobic autolysis.

Activation of Autolysis by —SH Compounds—In the following series of experiments we have added cysteine and other thiol compounds in order to determine their effects upon the over-all process of tissue digestion under fixed conditions of pH.

As before, digests were maintained at the initial pH levels throughout the digestion period by making the necessary adjustments at frequent intervals during the first 2 days. Nitroprusside tests were made from day to day. The naturally occurring reaction usually persists for about 2 days or less, while in digests to which cysteine has been added the reaction may persist several days longer.

The graph of a typical series resembles very closely the curves of control and N_2 gas as shown in Fig. 1. The addition of cysteine to the liver increases the speed and extent of digestion beyond that of the control at all pH levels tried and for the period of observation. While the effect appears to be upon the final level of cleavage attained and in the 10 day period is indistinguishable from that of the addition of some fragile protein like hemoglobin, the experiments were not carried on long enough to determine whether equilibria had been approximated in all cases. This point will be discussed later.

Similar increases of autolysis were produced by thiocresol and thioacetic acid. Thioglycolic acid produced no increase of autolysis but inhibited the reaction in proportion to the amount added.

Inhibition of Autolysis by Oxidants—Certain common oxidizing agents very rapidly abolish the naturally occurring nitroprusside reaction for —SH of freshly ground tissue. In this category are KIO_3, KIO_4, I_2, and $CuSO_4$. The first three are believed to oxidize —SH to —S—S— compounds

directly. Copper sulfate is said to catalyze the oxidation by oxygen. These oxidants inhibit the autolytic process at all pH levels tried. The degree of inhibition is determined by the amount of the oxidant added, within narrow limits, but even a very large excess does not completely arrest digestion. Inhibition may be counteracted by the addition of cysteine.

Since these four compounds give very much the same picture, we shall present data from one of them, KIO$_3$, as typical.

Effect of KIO$_3$—At pH 4 a preliminary trial showed that 0.75 cc. of 0.2 M KIO$_3$ in 30 minutes reduced the nitroprusside reaction in 100 cc. of the liver digest to a barely perceptible test. 1 cc. of the oxidant abolished the
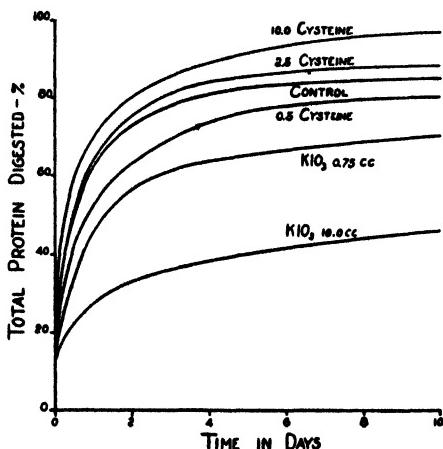
FIG. 2. The inhibition of autolysis at pH 4 by KIO$_3$ in an amount sufficient to abolish the —SH reaction, and in 10-fold excess; the reactivation by increasing increments of cysteine.

nitroprusside test completely. Cysteine increased autolysis over the control to a small extent. When increments of cysteine were added to digests in which the —SH reaction had been abolished, digestion was increased in proportion to the amount of cysteine added. In such digests the nitroprusside reaction was brilliant and persisted for 2 days or more. These results are shown in Fig. 2.

Certain other oxidants, when added to liver digests in amounts equivalent to KIO$_3$, have no effect upon the autolysis and do not abolish the nitroprusside reaction. The compounds in this category tried were KClO$_3$, KClO$_4$, and K$_2$S$_2$O$_8$.

There can be no doubt that there is a close correlation between the presence of —SH in a tissue digest and the speed and extent of autolysis at-

tained in limited periods such as 5 or 10 days. It is important, however, to know whether equilibria are being approached in 10 days in all digests, and whether these equilibria are artifacts due to destruction of the enzymes present or not. At pH 4 autolysis is ordinarily so rapid that digestion curves level off before the 10th day and represent substantially the final value of proteolysis. Further, it has been established (20) that the enzymes remain active for several weeks under control conditions, so that cessation of digestion indicates that all of the available substrate proteins have been digested, rather than that the enzymes have become inactive.

In the following prolonged experiment we have studied the behavior of cathepsin in digests treated with sufficient KIO_3 to remove immediately
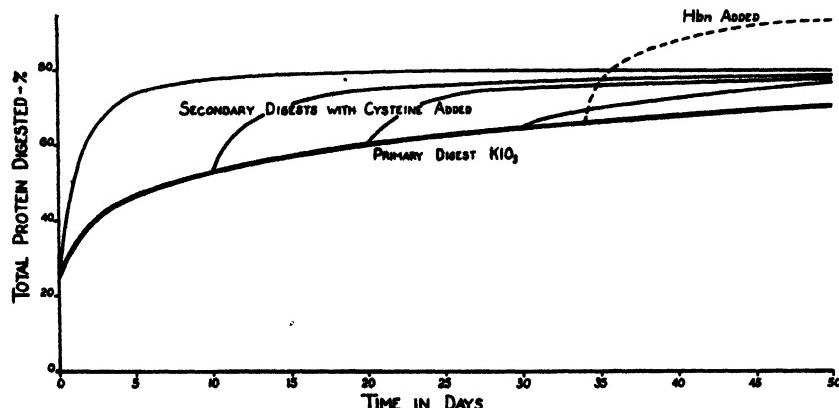
FIG. 3. The heavy curve represents autolysis inhibited by sufficient KIO_3 to abolish the —SH reaction. The lighter curves are for secondary digests activated at the times indicated by cysteine. The dotted curve represents the digestion of hemoglobin without cysteine activation. Activated and oxidized digests approach he same final level of digestion at different rates.
t

the nitroprusside reaction. The procedure was that previously described (20). A large 20 per cent liver hash was adjusted to pH 4. Sufficient KIO_3 was added to abolish the —SH test. From this primary digest were removed 100 cc. aliquots; they were treated with 2.5 cc. of 0.2 M cysteine solution and allowed to continue digesting. All digests were maintained at pH 4 throughout the experiment.

On the 34th day a secondary digest was set up with hemoglobin added instead of cysteine as an additional check on enzyme activity. The enzyme was found to be still active to hemoglobin (Fig. 3).

In this experiment about 80 per cent of the total liver N_2 was solubilized in the presence of cysteine. In the first secondary digest activated immediately, equilibrium was approximated in 10 days. There was very slow digestion following that point and up to 20 days, at which time the reaction

had become stationary. Later secondary digests did not quite attain the level of the first one, but the difference is not great enough to be certainly significant. All the secondary digests *approximate* 75 to 80 per cent of the total N_2 solubilized.

The primary digest proceeded very slowly but did not level off in the 50 day period. It is fair to conclude that digestion goes on more slowly in the absence of —SH but to the same ultimate level, if the time allotted is sufficiently long. From the slope of the later secondary digests it is apparent that the enzymes are gradually becoming less active and, in a very long digest of 60 days or more, might become wholly inactive. That all the cathepsin in the primary oxidized digest had not been destroyed, however, in 34 days exposure to the conditions set up is clear from the rapid digestion of hemoglobin added at that time, with no added cysteine.

DISCUSSION

So far as the over-all digestion of hog liver tissue is concerned, there can be no question but that the reducing level set up or indicated by cysteine leads to more rapid autolysis. The removal of cysteine as determined by the nitroprusside reaction, by such an oxidant as KIO_3, or merely by providing a high level of oxygen tension, decreases the rate of autolysis. In an extended period, however, the two reactions appear to approach an identical level of protein cleavage, as measured by soluble nitrogen and the tyrosine reaction.

Without more extensive data it will not be profitable to attempt positive interpretations at this time. Suffice it to point out that our results suggest the presence of two enzymes concerned in the primary cleavage of the tissue proteins. One of these is active whether cysteine is present or not, and in the absence of the activator autolysis goes on slowly to reach a cleavage level determined by the active masses of substrate and products. The second enzyme requires sulfhydryl activation. When this is provided, digestion is much more rapid, but the same final degree of cleavage is attained. Thus the effect of cysteine is to produce more active proteinase in the mixture rather than more available protein. This interpretation is in harmony with the results obtained by Fruton, Irving, and Bergmann working with small molecule, specific substrates, from which they conclude that splenic cathepsin is a multiple proteinase, some of whose factors require sulfhydryl activation, and some of which do not. It is also probable that the cathepsin prepared by Anson from spleen, which required no activation to digest hemoglobin, is a single member of this complex.

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CARBONIC ANHYDRASE

II. ZINC IN ITS RELATION TO CARBONIC ANHYDRASE ACTIVATION AND INACTIVATION

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This is a report of an investigation begun as an inquiry into the extent to which carbonic anhydrase can be affected, *in vitro*, by principles of known effect on respiration and circulation *in vivo*. A marked intensification of carbonic anhydrase activity was observed in the presence of adrenalin. The tracing of this effect to the contained catechol grouping and its interrelations with zinc led to the further developments described.

EXPERIMENTAL

Methods—Use was made of the Philpot technique for following the rate of carbon dioxide hydration, in terms of the time required for a specified change in pH in a carbonate-bicarbonate mixture undergoing saturation with streaming carbon dioxide at $0°$ (1, 2). Under the conditions controlling the measurements, 81 ± 3 seconds were required for the specified amount of hydration in the absence of modification by added enzyme or other substance. A single preparation of carbonic anhydrase was used, made by the "chloroform method" of Meldrum and Roughton (3). This preparation retained its initial activity unmodified throughout the investigation.[1] The substances tested for collateral effect were added to the reaction mixture as solutions in 0.00263 M sodium bicarbonate, along with sufficient 0.1 M NaOH or HCl to prevent substantial alteration in pH. The total volume was kept at 11 cc. An atmosphere of carbon dioxide was maintained during the solution, addition, etc., of the hydroxyphenols.

Effects of Adrenalin, Benzedrine, Ephedrine, and Paredrine—Only adrenalin, of these sympathomimetic drugs of related structure, had intensifying effect (Table I). The extent of the effect approximated that produced by histamine but was not attributable to the amino group which was, of

[1] Stored at ice box temperature, with the ordinary precautions against contamination, etc., the initially observed activity of this extract has persisted without significant deterioration for 16 months. Bakker (4) also found carbonic anhydrase to be a remarkably stable substance. The activity of his lens extracts was reported to be unchanged after storage in the ice box for a year. A highly purified preparation stored at room temperature in high dilution was found by Scott and Mendive (5) to lose 95 per cent of its activity within 24 hours. The loss did not occur if dilution was made with 0.05 per cent peptone instead of water.

TABLE I

Catechol Group in Adrenalin As Source of Enhancing Effect on Carbonic Anhydrase Activity; Relation of Effect to That Produced by Histamine, Pyrophosphate, and Diethyldithiocarbamate; Inhibition of Effect by Added Zinc

Substance added	Concentration	Hydration time		
		No enzyme (blank)	Enzyme present*	Enzyme + added zinc†
	mM per l.	sec.	sec.	sec.
None, 0.01 cc. enzyme.................		81 ± 3	33 ± 1	36 ± 1
" 0.02 " "			21	
" 0.03 " "			15	
" 0.005 " "			52	
Adrenalin, Parke Davis.................	0.45	78	18	44
"	0.045	78	22	38
Benzedrine, Smith, Kline and French ..	1.0	89	35	
Ephedrine, Mallinckrodt.............	1.0	87	37	
Paredrine, Smith, Kline and French..	1.0	88	36	
Catechol............................	0.5	82	18	31
"	0.1	80	19	
Pyrogallol...........................	0.5	81	19	31
Hydroquinone..........	1.0	81	34	
Resorcinol	1.0	80	32	
Phloroglucinol........	1.0	79	30	
Digallic acid.	1.0	80	40	35‡
Histamine............................	0.08	83	21	35
"	0.045	79	22	
Orthophosphate....	0.67	81	32	
Metaphosphate......	0.5	76	20	24
Pyrophosphate	0.5	78	16	15
"	0.05	78	24	28
Borate...........	1.0	82	36	
Cysteine	0.1	87	16	34
Sulfide................	<0.1§	77	62	
"	<0.02§	76	43	47
Diethyldithiocarbamate	0.1	76	20	48‡
Dithizone....	0.004	79	49	57
Sulfanilamide. ....,.	0.012	74	40	41

* 0.01 cc. except where otherwise specified.

† In a final concentration of 0.025 mM per liter, except with the digallic acid in which 0.5 mM per liter was added.

‡ Formation of a visible precipitate.

§ A small, undetermined percentage of the added sulfide was swept out of the titration mixture by the CO₂ stream.

course, common to the series. The amino group introduced the slight lengthening apparent in the blank hydration time. Phenol and glycerol (6 to 7 mM per liter) and freshly dissolved and neutralized ascorbic acid (1.0 mM per liter) were without effect.

Effects of Catechol, Hydroquinone, and Resorcinol—These compounds contain two hydroxy groups attached to a benzene ring, the first in the ortho relationship present in adrenalin, the second in a para, and the third in a meta relationship. Catechol and the related pyrogallol exerted an intensifying action equivalent to that produced by adrenalin. Hydroquinone, resorcinol, and the related phloroglucinol were without effect. Digallic acid, containing the catechol grouping but without the catechol effect on the solubility of zinc hydroxide reported in Table III, caused a diminution of activity.

Prevention of Catechol Effect by Added Zinc—Zinc in a concentration of 0.025 mM per liter, having no substantial direct effect on the enzyme, exerted the same blocking action against intensification of carbonic anhydrase activity by added catechol and pyrogallol that had been earlier observed against histamine and cysteine (2, 6).

Effects of Ortho-, Meta-, and Pyrophosphate—The orthophosphate ion produced no shortening of the hydration time either in the presence or the absence of carbonic anhydrase. Borate also was without significant effect. The pyrophosphate ion in a concentration of 0.5 mM per liter had an intensifying effect on carbonic anhydrase action equaling that contributed by peptone broth and by heated plasma (2, 5-7). The related metaphosphate ion was less active in this direction. The effect was blocked by added zinc provided the intensifying agents were not present in a ratio, to the added zinc, markedly exceeding 2:1.

Effects of Sulfide, Cysteine, Diethyldithiocarbamate, Dithizone, and Sulfanilamide in Their Relation to Zinc—Cysteine had an intensifying action exceeding that of pyrophosphate. The contrasted diminishing action of sulfide approached that exerted by sulfanilamide and dithizone. The intensifying action of diethyldithiocarbamate was reversed, into an inhibition, by added zinc. The latter effect was accompanied by evidence of precipitation. Sulfide, dithizone, and sulfanilamide were fully as inhibitory in the presence of added zinc as in its absence.

Extent to Which Effects of Histamine, Catechol, and Pyrophosphate Were Additive—Additions of histamine and catechol produced a greater degree of activation than was realizable through addition of either substance alone (Table II). Additive effect was less apparent in the combinations with pyrophosphate.

Extent to Which Sulfanilamide and Sulfide Prevented Activation by Catechol, Pyrophosphate, and Cysteine—The activity of carbonic anhydrase plus

sulfanilamide was increased to about the same extent by catechol as the activity of carbonic anhydrase without sulfanilamide addition (Table III).

TABLE II

Summation of Effects of Catechol and Histamine; Non-Summation with Pyrophosphate

Concentration of added			Hydration time, enzyme present[*]	Indicated enhancement of activity[†]
Catechol	Histamine	Pyrophosphate		
mM per l.	*mM per l.*	*mM per l.*	*sec.*	*per cent*
0	0	0	51	
0.5	0	0	30	200
0	0.32	0	33	155
0.5	0.32	0	24	320
0	0	1.0	23	370
0	0.32	1.0	22	410
0.5	0	1.0	23	370

[*] 0.005 cc. of the solution described for Table I.

[†] Activity $= 1/V_{unit} = (1/V)\,(1/t - 1/t_0)/(1/t_0)$, where V is the volume in cc. of the enzyme preparation added and t and t_0 are the observed hydration times in seconds in the presence of that volume of added enzyme and in the compared blank. V_{unit} is the volume of enzyme giving a hydration time equal to one-half the blank (1, 2).

TABLE III

Combined Intensifying and Inhibiting Effects

Concentration of added inhibitor		Activity in enzyme-inhibited mixture	Increase[*] in activity produced by additions of 0.1 mM per liter of		
Sulfanilamide	Sulfide		Catechol	Pyrophosphate	Cysteine
mM per l.	*mM per l.*	*units[†]*	*per cent*	*per cent*	*per cent*
0	0	160	100	130	240
0.01	0	90	110	140	190
0.03	0	45	100	70	120
0	0.01	110	20	50	90
0	0.03	60	0	0	80

[*] Virtually the same changes in activity were found whether the catechol, pyrophosphate, and cysteine were added before or after the sulfanilamide or sulfide. The figures are given in terms of increase from the base-line purely for convenience of presentation.

[†] See [†] foot-note of Table II.

Conversely, the activity of carbonic anhydrase plus catechol was decreased to about the same extent by sulfanilamide as the activity manifest in the absence of catechol addition. Comparably balanced action was apparent

between sulfanilamide and cysteine and also between sulfanilamide and pyrophosphate although to a less consistent extent.

In contrast, carbonic anhydrase plus sulfide appeared to be only partially susceptible to activation by cysteine and very nearly completely insusceptible to activation by catechol and pyrophosphate. Inhibition by

TABLE IV

Precipitability of Zinc from Solutions Containing Substances with Intensifying and Inhibitory Effect on Carbonic Anhydrase Activity

Substance*	Volume of precipitate formed at pH 7.4 with		
	No additions	Added phosphate (2.5 mM per liter)	Added sulfide (1.2 mM per liter)
	cc.	*cc.*	*cc.*
None........................	0.10	0.20	0.20
Catechol....................	0	0.04	0.05
Hydroquinone...............	0.13	0.20	0.12
Resorcinol..................	0.20	0.20	0.10
Pyrogallol..................	0	0	0.01
Phloroglucinol.............	0.60†	0.20	0.10
Histamine..................	0	0.13	0.03
Cysteine...................	0	0	0
Pyrophosphate	0	0	0.03
Peptone...................	0.20	0.18	0.15
Sulfanilamide.............	0.40†‡	0.18	0.18

* The final concentration of each added substance was 20 mM per liter except for the peptone which was 0.18 per cent. Each mixture was made up to a total volume of 10 cc. and contained 3.75 mM of $ZnSO_4$ per liter.

† Precipitate loose and gelatinous.

‡ No sulfanilamide was carried into the precipitate.

sulfide appeared to exclude opportunity for activation by agents other than cysteine.

Extent to Which Substances with Intensifying Effect on Carbonic Anhydrase Action Tended to Stabilize Zinc against Precipitation—A series of mixtures containing zinc sulfate and the various added substances listed in Table IV in the volume and concentration there stated was adjusted to pH 7.4, allowed to stand until flocculation, when present, was complete, and then placed in the centrifuge. The indicated volumes of sediment are not intended to be other than a rough gage of the effects of the added substances on precipitability.

Catechol was clearly differentiated from hydroquinone and resorcinol

in its preventive action against zinc precipitation, as was pyrogallol from phloroglucinol and pyrophosphate from orthophosphate. Added phosphate and sulfide tended to diminish the preventive effect. Histamine and cysteine had a preventive action not shared by peptone.

DISCUSSION

It is apparent from the relations presented in Table I that amounts of zinc in themselves insufficient to produce substantial inhibition of carbonic anhydrase activity successfully prevented the enhancement of activity obtained, in the absence of added zinc, by added catechol, histamine, cysteine, and pyrophosphate *but failed to prevent the contrasted inhibitions by sulfide, dithizone, and sulfanilamide*. It is further apparent, from Table IV, that a limited parallelism exists between the extent to which catechol, cysteine, and pyrophosphate produced enhancement of carbonic anhydrase activity and the extent to which stabilization was exerted against the zinc precipitations there described.

These findings suggest a probability of action against zinc, but not against a zinc grouping in the enzyme itself. The action would be, rather, against traces of zinc or its equivalent extraneous to the enzyme principle. How such an action could have associated effects on carbonic anhydrase activity is made clear by the experiment with diethyldithiocarbamate. In this experiment, precipitate formation by an amount of zinc with no substantial, direct inhibiting action and an amount of diethyldithiocarbamate, which by itself produced an intensification of activity, together caused an unmistakable loss of activity. The existence of adsorption affinities, between the enzyme and the precipitating substance, could easily cause loss of activity on addition of substances favoring precipitation, and stabilization or increase in activity on addition of substances preventing or reversing precipitation. An action of this sort has been reported by Hove, Elvehjem, and Hart (8). These workers found a reduction in carbonic anhydrase activity following additions of preformed zinc dithizonate and suggested a mechanism of adsorption of enzyme substance to the colloidal metal dithizonate particles as a possible explanation for the loss. The effect was not specific for added zinc dithizonate but was elicited also by other, related metal dithizonates. Nor is zinc the only metal blocking the intensifying action of histamine, cysteine, etc. (6).

Hove, Elvehjem, and Hart also observed, for zinc, an implementing action on amino acid intensification of intestinal phosphatase activity (9) curiously contradirectional to the blocking action exerted against amino acid intensification of carbonic anhydrase activity.

The inability of histamine to produce substantial increase in intensifying effect over and above that exerted by pyrophosphate simultaneously pres-

ent, as reported in Table II, has bearing, along with the observation of antagonism between phosphate and histamine indicated in Table IV, on the controversy between Kiese (10) and Leiner and Leiner (11) with respect to histamine as an activator of carbonic anhydrase. Kiese's failure to observe the effect was ascribed by Leiner and Leiner to the use of a method of appraisal requiring a high concentration of pyrophosphate buffer. Pyrophosphate in high concentration has also been reported to interfere with the combination of histamine and carbon dioxide into carbamate (12).

The inhibiting action of neither sulfide nor sulfanilamide can be presumed to be exerted on zinc adventitiously present, since it is neither counteracted nor substantially augmented (through induced coprecipitation) by added zinc. Sulfanilamide was, furthermore, not observed to be carried down with precipitating zinc at pH 7.4.

SUMMARY

1. The activity of partially purified preparations of carbonic anhydrase, as appraised by the Philpot method of titration, was intensified by adrenalin, but not by benzedrine, ephedrine, or paredrine.

2. An equivalent intensification of activity was exerted by catechol and pyrogallol but not by hydroquinone, resorcinol, phloroglucinol, phenol, glycerol, or ascorbic acid.

3. Marked intensification was produced by pyrophosphate, cysteine, and diethyldithiocarbamate. Some intensification was apparent with metaphosphate and none with orthophosphate or borate. A degree of inhibition exceeding that exerted by sulfide was observed with dithizone and with sulfanilamide.

4. The intensifying actions were, without exception, blocked by added zinc. That of diethyldithiocarbamate was changed, following addition of zinc, into a marked inhibition. Zinc together with dithizone also was more inhibitory than dithizone alone.

5. The intensifying actions of catechol and histamine were additive. The intensification produced by pyrophosphate was not substantially increased by additions of either catechol or histamine. The inhibiting action of sulfanilamide was competitive with the intensifying action of catechol, cysteine, and pyrophosphate. The one action did not exclude the production of the other. Inhibition through sulfide tended to exclude the production of intensification by catechol and pyrophosphate and lessened the extent of intensification produced by cysteine.

6. The differentiations in intensifying action between catechol and hydroquinone, pyrogallol and phloroglucinol, pyrophosphate and orthophosphate, cysteine and sulfide were paralleled by a protective effect against zinc precipitation at pH 7.4.

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STUDIES ON THE CALCIUM-PROTEIN RELATIONSHIP WITH THE AID OF THE ULTRACENTRIFUGE*

I. OBSERVATIONS ON CALCIUM CASEINATE SOLUTIONS

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It is recognized that calcium in protein-containing solutions exists in both diffusible and non-diffusible states, and that at least two forms of calcium are in equilibrium with each other. The quantitative aspects of this relationship have been studied by a variety of methods such as ultrafiltration, the McLean-Hastings frog heart technique, and by direct correlation of data for protein and calcium. An outstanding contribution to the subject in recent years was made by McLean and Hastings (1), who showed that the ionization of calcium in serum and solutions of purified proteins is determined by a relationship between calcium and protein which can be expressed to a first approximation by a simple mass law equation. The work reported in this paper is designed to test the validity of this conclusion and to investigate certain aspects of the problem not accessible to previous methods.

The ultracentrifuge affords a physicochemical approach to this problem. With this method, a calcium-protein solution is exposed to centrifugal forces sufficient to produce gradients in the protein and calcium concentrations. A study of these gradients provides a method for determining the diffusible calcium concentration and the amount of calcium bound to protein.

Methods

The Ultracentrifuge—The driving mechanism was similar to that described by Beams, Linke, and Sommer (2). The rotor was designed by Masket (3) and is a modification of the Bauer and Pickles quantity type (4). The holes in this rotor were bored at a 10° angle and thus have an advantage over the 45° angle type because there is less distance for a molecule to sediment before reaching the Lusteroid test-tube wall farthest from the axis. The loaded rotor, with a capacity of 66 cc. of solution, was kept at 5° overnight and was placed in the vacuum chamber just before the run was begun. A vacuum of 0.5 μ or less was maintained during the run

with a Megavac and an oil diffusion pump. The rotor was run at 1000 revolutions per second, exerting a mean of 200,000 times gravity. The casein solutions were fractionated with the apparatus described by Hughes, Pickles, and Horsefall (5).

Casein (Pfanstiehl, highest purity) was dissolved in either 0.2 N NaOH-H₂O or 0.2 N NaOH-0.9 per cent NaCl mixtures. The hydrogen ion concentration was measured with a glass electrode. Total nitrogen was determined by the micro-Kjeldahl procedure and was converted to protein by the factor 6.3. Calcium was determined in trichloroacetic acid filtrates according to the modified procedure of Halverson and Bergeim (6) as described by Peters and Van Slyke (7). The water concentration was determined in a large number of casein solutions of varying nitrogen concentration after drying for 48 hours at 105°. These results were plotted and the equation $H_2O = 99.8 - 0.0021 N$ was calculated from the graph. This formula was used subsequently for estimating the percentage concentration of water.

Theoretical Considerations

According to the work of McLean and Hastings, calcium proteinate (CaProt), in solution, behaves as a weak electrolyte whose dissociation, to a first approximation, can be expressed by the law of mass action

$$(1) \qquad \frac{[Ca^{++}] \times [Prot^-]}{[CaProt]} = K_{CaProt}$$

McLean (8) subsequently pointed out that the assumption that protein behaves as a series of divalent ions is unnecessary, and that the equations may equally well be written, for example,

$$\frac{[Ca^{++}][Prot^-]}{[CaProt^+]} = K_{CaProt^-}$$

with

$$[CaProt^+] \rightleftarrows [Ca^{++}] + [Prot^-]$$

Since it is impossible to determine protein ion concentration directly, protein ion concentration can be eliminated by substitution from

$$(2) \qquad [Total\ Prot] = [Prot^-] + [CaProt]$$

which yields on rearrangement the equation

$$(3) \qquad \frac{[Total\ Prot]}{[CaProt]} = 1 + \frac{K}{[Ca^{++}]}$$

To make use of the experimentally determined value of total protein in gm. per 100 gm. of H_2O, it is necessary to assume that

(4) $$[\text{Total Prot}] = f \times \text{total Prot}$$

where f is a constant factor converting protein as gm. per 100 gm. of H_2O to protein as moles per kilo of H_2O, and is understood as the conversion factor at $[Ca^{++}] = \infty$. The mass law then takes the form

(5) $$\frac{\text{Total Prot}}{[\text{CaProt}]} = \frac{1}{f}\left(1 + \frac{K}{[Ca^{++}]}\right)$$

which is essentially that given by Greenberg, Larson, and Tufts (9).

The application of the law of mass action (Equation 5) depends on the knowledge of the value of the conversion factor f. While recognizing the weakness of their procedure, McLean and Hastings used factors obtained from the titration of protein with base in order to convert protein, in terms of gm., to moles. It is possible to determine f by an extrapolation from a range of finite and small concentrations of Ca^{++} to the conditions at $Ca^{++} = \infty$. Therefore, a preliminary, but necessary, step in the determination of f is an accurate determination of $[Ca^{++}]$ over as wide a range as possible. Both of these conditions have been fulfilled in the work here reported.

The possible interpretations of Equation 5 are given as follows:

When $[Ca^{++}]$ Is Constant—If the $[Ca^{++}]$ is constant in a set of solutions, it follows from Equation 5 that a linear relationship exists between [CaProt] and total protein and may be expressed as

(6) $$\text{CaProt} = m \times \text{total protein}$$

where

(7) $$\frac{1}{m} = \frac{1}{f}\left(1 + \frac{K}{[Ca^{++}]}\right) = \frac{\text{total Prot}}{[\text{CaProt}]}$$

Assuming

(8) $$[\text{Total Ca}] = [\text{CaProt}] + [Ca^{++}]$$

it follows from Equation 6 that

(9) $$[\text{Total Ca}] = m \times \text{total protein} + [Ca^{++}]$$

Thus, $[Ca^{++}]$ can be determined graphically as the intercept on the [total Ca] axis of a plot of Equation 9 where m is the slope of the same plot.

When $[Ca^{++}]$ Is Not Constant—When $[Ca^{++}]$ is varied in a set of solutions, it follows from Equation 5 that a linear relationship exists between (total Prot)/[CaProt] = $1/m$ and $1/[Ca^{++}]$. In a plot of Equation 5 with variable $[Ca^{++}]$, K/f is the slope and $1/f$ the intercept of the line extrapolated to the $1/m$ axis; that is, f is determined for the condition $[Ca^{++}] = \infty$. In this way, the use of titration data has been avoided and any assumption as to valency made unnecessary.

The theoretical considerations have dealt with solutions containing calcium salts and pure protein. In body fluids, it is known that diffusible calcium may exist in a dissociated and undissociated state. In such solutions Equations 8 and 9 can be generalized to

(8, a)   [Total Ca] = [CaProt] + [diffusible Ca]

(9, a)   [Total Ca] = m × total Prot + [diffusible Ca]

where [diffusible Ca] = [Ca^{++}] + [CaX] and [CaX] represents the concentration of un-ionized diffusible Ca. These generalizations are valid because the presence of [CaX] cannot affect the equilibrium constant K_{CaProt} and the equations derived from the mass law. In the presence of [CaX], the intercept in a plot of Equation 9, a would include both ionized and un-ionized Ca. By rearrangement of Equation 7, the equation

(10) $$[Ca^{++}] = \frac{mK}{f - m}$$

can be used for determining [Ca^{++}] from the slope m, if f and K are known.

Dissociation of Calcium Caseinate—The data presented in graphic form in Fig. 1 represent a typical experiment in which a casein solution with added calcium chloride was centrifuged and fractionated into four equal portions. It is clear that there is a linear relationship between total calcium and total protein. It follows from this diagram that the intercept with the calcium axis is the [Ca^{++}] or diffusible calcium of the solution studied. The slope of the line (m) determines the amount of bound calcium (CaProt). It is seen that the ultracentrifuge makes it possible to obtain a series of solutions whose calcium and protein concentrations vary markedly from the original. If Ca^{++} is assumed to be constant in each fraction, it follows that Fig. 1 represents a plot of Equation 9, which is an expression of the law of mass action.

Two series of casein solutions dissolved in NaOH-NaCl and NaOH-H$_2$O, respectively, of constant protein and varying CaCl$_2$ concentrations were centrifuged, fractionated, and the fractions analyzed for calcium and protein. These data are plotted and analyzed in Fig. 2 and Table I. Owing to the insolubility of calcium caseinate, the ability to increase the calcium ion concentration is limited. These experiments make it possible to determine the dissociation constant of calcium caseinate and to obtain the factor f for converting casein from gm. to mM.

If a plot is made for $1/m$ against $1/[Ca^{++}]$ (Fig. 3) according to the procedure used by Greenberg, Larson, and Tufts (9) in which Equation 7 is used, linear relationships are obtained for the two solutions. No explanation can be given for the deviation from linearity in the aqueous solution of lowest Ca^{++} concentration, a phenomenon noted in repeated
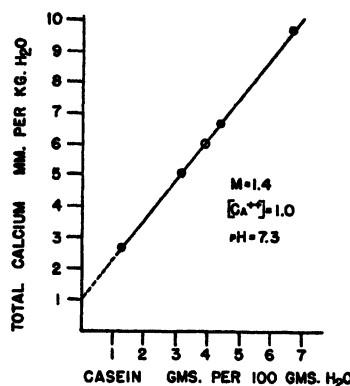
FIG. 1. A typical plot of values for a casein-$CaCl_2$ solution centrifuged at 1000 revolutions per second for 2 hours. ● represents four fractions of the ultracentrifuged solution and O represents the values for the original solution. The slope of the line is designated by m, and the intercept at the calcium axis is assumed to be the calcium ion concentration.
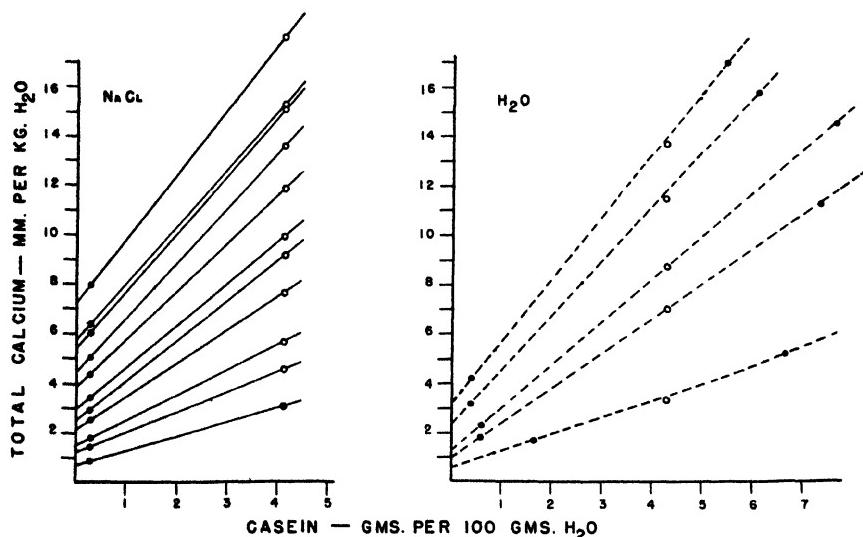
FIG. 2. The effect of varying the calcium concentrations of casein dissolved in 0.9 per cent NaCl and aqueous solutions (pH 7.2). The saline solutions were centrifuged at 1000 revolutions per second for 5 hours, and the top fraction and original analyzed. The aqueous solutions were centrifuged for the same time and divided into two portions for analysis.

<div align="center">

TABLE I

Effect of Varying Total Calcium Concentration of Casein Solutions (4.0 Gm.
per 100 Gm. of H_2O)

</div>

	Original Ca concentration	$[Ca^{++}]$	Slope (m)
	mm per kg. H_2O	*mm per kg. H_2O*	
NaOH-NaCl	3.1	0.7	0.57
	4.6	1.2	0.81
	5.7	1.6	1.00
	7.7	2.2	1.35
	9.2	2.7	1.58
	10.0	3.0	1.67
	11.9	3.9	1.94
	13.6	4.4	2.22
	15.1	5.4	2.28
	15.3	5.8	2.28
	18.0	7.2	2.62
NaOH-H_2O	3.3	0.7	0.66
	7.0	1.1	1.37
	8.8	1.4	1.70
	11.5	2.4	2.13
	13.6	3.2	2.46
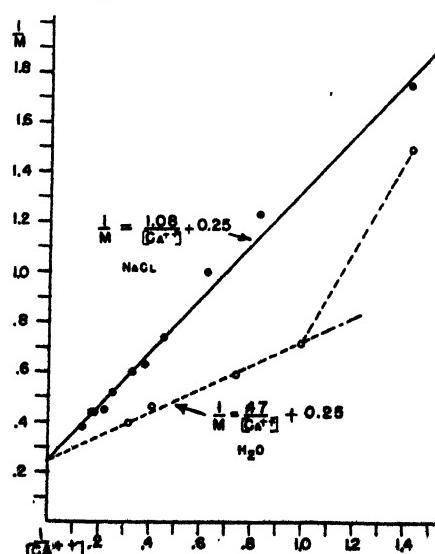
FIG. 3. A plot of values for the reciprocals of the values for the slopes (m) and the calcium ion concentrations of the data in Fig. 2.

experiments. The increased ionic strength due to the presence of NaCl obviously increases the dissociation of calcium caseinate, as is shown by the values of 1.08 and 0.47 for the respective slopes for the saline and water solutions in Fig. 3. The intercept on the $1/m$ axis represents the reciprocal of the factor f. Since both lines have the same intercept (0.25) at the $1/m$ axis, it must be concluded that only at infinite concentrations of calcium ion will protein exert its maximum combining power in aqueous or saline solutions.

Since $K = f \times K/f$, it follows that $K_{CaCasein}$ in H_2O equals $4.0 \times 0.47 = 1.88$ and $K_{CaCasein}$ in saline equals $4.0 \times 1.08 = 4.32$, or pK equals 2.73 and 2.36, respectively.[1]

Using the factor f of Pertzoff and Carpenter (10), McLean and Hastings obtained the $pK_{CaCasein}$ of 2.38 with the frog heart procedure. If the value of f obtained in the present experiment was used, the pK for calcium

TABLE II

Effect of Varying Casein (Dissolved in NaOH-H_2O) Concentration on $pK_{CaCasein}$

All solutions were at pH 7.22; centrifuged at 1000 revolutions per second for 2½ hours and fractionated into four portions.

Total protein	Total Ca	Ca++	Slope (*m*)	$pK_{CaCasein}$
gm. per 100 gm. H_2O	*mM per kg. H_2O*	*mM per kg. H_2O*		
2.2	6.0	1.7	1.94	2.75
2.6	5.9	1.2	1.76	2.81
3.1	6.0	1.2	1.65	2.77
4.2	6.0	0.6	1.30	2.90
6.0	5.8	0.5	0.95	2.80

caseinate approximated the value of 2.23 which was found by Weir and Hastings (11) after equilibration studies.

Ionization of Calcium Caseinate in Presence of Varying Quantities of Casein—From the theoretical considerations presented, the $pK_{CaCasein}$ should not vary with the protein concentration. In Table II the data are summarized for a series of solutions in which the calcium and hydrogen ion concentrations are kept constant and the protein concentration is varied. The plot of the calcium and protein values shows a linear

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[1] The calculation of a pK value for a single centrifuged solution depends on the use of Equation 10 in the form $K = Ca^{++} ((f/m) - 1)$. For example, if $[Ca^{++}] = 0.7$ and $m = 0.57$, $K = 0.7 ((4.0/0.57) - 1) \times 10^{-3} = 4.2 \times 10^{-3}$, pK $= 3 - \log_{10} 4.2 = 2.38$. The values for f and m throughout the paper are given for 10 gm. of protein, since protein is expressed in gm. per 100 gm. of H_2O, while Ca is expressed in mM per kilo of H_2O. In order to convert calcium to moles, it is necessary to use the factor 10^{-3}.

relationship for the respective solutions and the [Ca^{++}] are obtained from the intercepts at the Ca axis. The values for pK$_{CaCasein}$ in these experiments are consistently uniform and are not affected by large or small concentrations of protein.

Ionization of Calcium Caseinate in Presence of Varying Quantities of Sodium Chloride—The effect of varying the ionic strength on the [Ca^{++}]

<div align="center">TABLE III</div>

Effect of Varying Sodium Chloride Concentration of Calcium Caseinate Solutions on Ca^{++} and pK$_{CaCasein}$

All solutions were at pH 7.2; centrifuged at 1000 revolutions per second, for 2½ hours and divided into two fractions. The total protein was 3.7 gm. per 100 gm. of H$_2$O and the total Ca 5.2 mM per kilo of H$_2$O.

NaCl	Ca^{++}	Slope (*m*)	pK$_{CaCasein}$
gm. per cent	*mM per kg. H$_2$O*		
0.0	1.5	1.08	2.39
0.3	1.6	1.00	2.32
0.6	1.6	1.00	2.32
0.9	2.3	0.80	2.04
1.2	2.4	0.75	1.98
1.5	2.4	0.75	1.98

<div align="center">TABLE IV</div>

Results after Dilution of Calcium Caseinate Solution with 0.9 Per Cent NaCl

Each sample was centrifuged at 1000 revolutions per second and fractionated into four portions; the first four solutions were centrifuged 2 hours and the last for 1½ hours; all pH values at 7.2.

Fraction No.	Total protein	Total Ca	Ca^{++}	Slope (*m*)	pK$_{CaCasein}$
	gm. per 100 gm. H$_2$O	*mM per kg. H$_2$O*	*mM per kg. H$_2$O*		
1 (Original)	3.96	5.16	1.5	0.95	2.32
2	3.50	4.69	1.6	0.88	2.25
3	2.90	3.74	1.4	0.89	2.31
4	2.00	2.58	1.0	0.71	2.34
5	1.23	1.66	0.8	0.60	2.34

and the pK$_{CaCasein}$ is shown in Table III. To aliquots of a stock solution of casein and CaCl$_2$ dissolved in NaOH-H$_2$O, sodium chloride was added in varying quantities. No change in the values of Ca^{++}, *m*, and pK are observed in the first three solutions containing 0, 0.3, and 0.6 per cent sodium chloride. Further increases in ionic strength yield greater [Ca^{++}] and smaller pK$_{CaCasein}$ values. The results are identical for the solutions containing 0.9, 1.2, and 1.5 per cent NaCl.

The $pK_{CaCasein}$ for the aqueous solution is 2.39, which is smaller than similar determinations obtained in previous experiments. This inconsistency is probably due to the lack of uniformity in the different batches of casein purchased. It was noted that the time of centrifugation necessary to obtain a small pellet and an appreciable concentration of casein in the bottom of the tube varied considerably with different batches. Nevertheless the relative changes are reliable, since the same casein solution was used in this experiment.

Effect of Dilution of Calcium Caseinate Solutions upon Ionization of Calcium—Table IV shows an excellent degree of constancy for $pK_{CaCasein}$
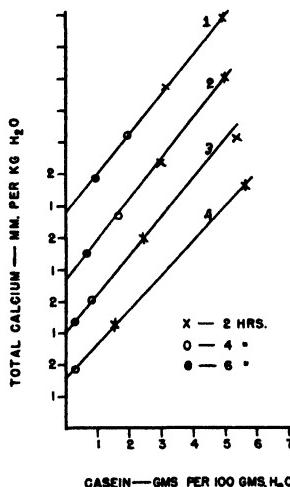
FIG. 4. Casein solution, adjusted to different pH values, centrifuged for 2, 4, and 6 hours. The top and bottom fractions were analyzed after 2 hours centrifugation and the top third was analyzed for the 4 and 6 hour samples. Curve 1, pH 7.48, m 1.3, Ca^{++} 0.7; Curve 2, pH 7.0, m 1.28, Ca^{++} 0.7; Curve 3, pH 6.52, m 1.18, Ca^{++} 1.0; Curve 4, pH 6.05, m 1.08, Ca^{++} 1.5.

as determined with a calcium caseinate solution after varying dilutions. Sufficient 0.9 per cent NaCl was added to approximate dilutions of 90, 70, 50, and 30 per cent of the original solution, thus avoiding any change in ionic strength in the respective solutions. These experiments in which the total protein and total calcium are varied proportionately present further evidence for the validity of the mass law as applied to calcium caseinate solutions. It should be noted that dilution of calcium caseinate cannot be considered as an inverse process to centrifugation despite the maintenance of a proportional decrease in the concentrations of total protein and total calcium because the $[Ca^{++}]$ and the ratio $[CaProt]/$ total Prot vary.

Effect of Time of Centrifugation—These experiments (Fig. 4) were per-performed to determine whether different periods of centrifugation affected the ratio [CaProt]/total Prot in calcium caseinate solutions (3.8 gm. of protein per 100 gm. of H₂O and 5.9 mM of Ca per kilo of H₂O) of different hydrogen ion concentrations. Since a linear relationship exists between all fractions of the solutions of identical pH, it follows that the ratio remains constant and from Equation 9 the [Ca⁺⁺] must also be constant. Hence it can be concluded that the time of centrifugation does not affect the nature of the equilibrium between calcium and casein.

Effect of pH on Ionization of Calcium Caseinate—A stock solution of calcium caseinate (NaOH-H₂O) with protein and calcium concentrations of 4.1 gm. per 100 gm. of H₂O and 5.6 mM per kilo of H₂O, respectively, was prepared. Aliquots of this solution were adjusted to different hydrogen ion concentrations by means of a few drops of strong NaOH or HCl. All these solutions were centrifuged for 2¼ hours and fractionated into six equal portions. The results are shown in graphic form in Fig. 5.

The [Ca⁺⁺] remains constant at about 1.8 mM over a wide range varying from pH 6.58 to 8.5. The highest and the lowest Ca⁺⁺ are obtained at 5.9 and 8.97, respectively. All of the solutions mentioned above exhibit linear relationships. The calcium caseinate solutions with pH values of 9.1 or greater deviate from linearity. Extrapolation of such lines would have no significance or justification.

Since all casein solutions were centrifuged the same length of time and were fractionated in the same manner, it is significant that the protein concentrations in the respective fractions vary in a definite pattern with changes in pH. It is clear that increased alkalinity affects the sedimentation of the casein molecule. The solution at pH 10.1 shows this phenomenon strikingly and exhibits a marked curvature of the plot of the different fractions. Evidence is presented to show that the effect is reversible to a great extent in the experiment in which a calcium-casein solution, kept at pH 10.1 in a cold room overnight, was adjusted to pH 7.4 shortly before centrifugation. Sedimentation of protein greatly increased, and the curvature which is markedly decreased approaches but does not quite attain linearity. No studies were carried out to determine whether these changes are associated with racemization or denaturation or both.

The effect of pH on the ionization of calcium in protein-containing solutions has been studied by a variety of methods which has resulted in differences of findings and interpretations. Ultrafiltration studies (12–14) indicate that the diffusible calcium increases with decreased pH. By means of equilibration studies, Weir and Hastings (11) found that the [Ca⁺⁺] decreased when the pH increased in casein solutions. Using the frog heart method, McLean and Hastings were unable to demonstrate any change

in [Ca^{++}] with change in pH. In most of these experiments the pH range was limited.

According to the modern theory, the protein molecule may be in an electrically neutral (zwitter ion), negatively charged, or positively charged
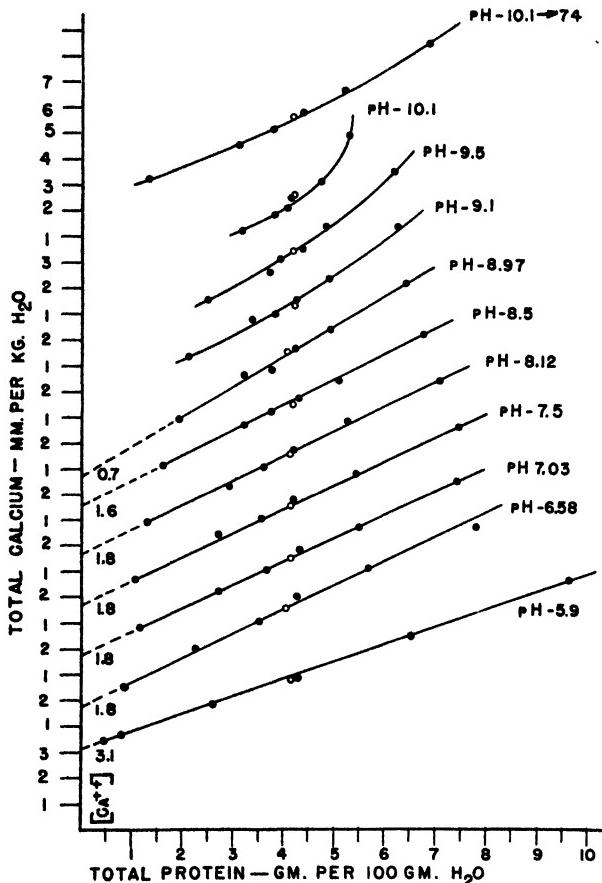
Fig. 5. A graph of calcium caseinate solutions at different hydrogen ion concentrations.

state, usually represented by the three respective formulae COO$^-$·R·NH$_3$$^+$, COO$^-$·R·NH$_2$, or COOH·R·NH$_3$$^+$. The concentration of charged molecules depends on the pH of the solution in relation to the isoelectric point. McLean[2] has evidence to show that calcium combines with the protein ion and the zwitter ion on the alkaline side of the isolectric point. He assumes

[2] McLean, F. C., personal communication.

that the total number of COO⁻ groups is constant and therefore f should remain constant within limited pH ranges. In their discussion of the ionization of calcium proteinate, McLean and Hastings cite evidence (15, 16) to show that calcium combines with protein on the acid side of the isoelectric point. If it is assumed that calcium reacts with the COO⁻ groups of the zwitter ions and is indifferent to the positive charge, it is possible to explain the preceding observation.

TABLE V

Effect of Changing pH on Dissociation of Calcium Caseinate ($NaOH-H_2O$)

The total calcium and total protein concentrations were 5.95 mM per kilo of H_2O and 4.0 gm. per 100 gm. of H_2O, respectively; the solutions were centrifuged for 2.5 hours at 1000 revolutions per second, and fractionated into four equal portions. $f = 4.0$.

pH	Ca	Slope (m)	$pK_{CaCasein}$*
	mM per kg. H_2O		
7.30	1.0	1.40	2.72
7.17	1.0	1.25	2.67
6.98	1.0	1.36	2.71
6.85	1.0	1.32	2.69
6.80	1.0	1.30	2.68
6.77	1.0	1.28	2.67
6.68	1.0	1.30	2.68
6.60	1.0	1.28	2.67
6.48	1.0	1.30	2.68
6.41	1.0	1.27	2.66
6.30	1.0	1.25	2.67
6.21	1.4	1.17	2.47
6.08	1.6	1.18	2.42
5.98	1.6	1.17	2.41
5.94	1.6	1.13	2.40

* Calculated from $K = Ca^{++} ((f/m) - 1)$.

A group of calcium caseinate ($NaOH-H_2O$) solutions was prepared in which the pH was varied in short intervals (Table V). The values for $[Ca^{++}]$,[3] m, and $pK_{CaCasein}$ are constant for eleven solutions showing slight variations in pH between 7.3 and 6.3. The change from 6.3 to 6.21 results in an abrupt increase in $[Ca^{++}]$. In view of the discussion concerning factor f, it is assumed that variations in the $[Ca^{++}]$ and m are to be attributed to a change in the value of K. In those solutions in which the $[Ca^{++}]$ is greater than 1.0 mM, there is a decrease in the value of $pK_{CaCasein}$.

[3] Despite the almost identical original values for the protein and calcium in the two solutions of Fig. 5 and Table V, the respective calcium ion concentrations show marked differences. This is probably due to the fact that two different batches of casein were used.

Effect of Citrate on Diffusible Calcium—Varying amounts of sodium citrate were added to calcium caseinate solutions ($NaOH-H_2O$) whose protein and calcium concentrations were 3.96 gm. per 100 gm. of H_2O
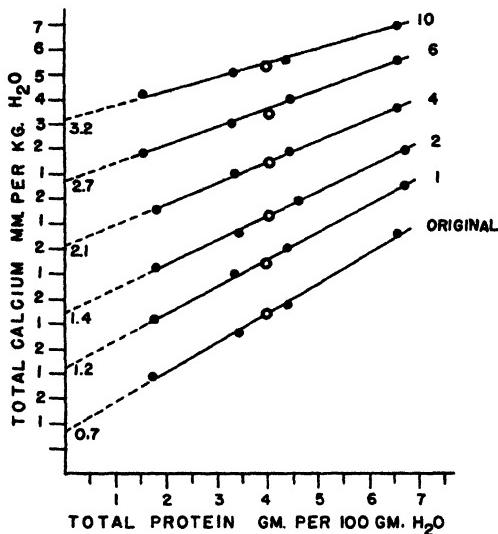
Fig. 6. Plots of ultracentrifuged casein solutions with constant total calcium and varying citrate concentrations. The figures at the intercept represent total diffusible calcium and the figures at the end of the lines show the concentrations of citrate in terms of mM per liter.

TABLE VI

Dissociation of Calcium Citrate in Solutions of Calcium Caseinate to Which Varying Amounts of Citrate Were Added

All solutions were maintained at pH 7.0. All concentrations are expressed in terms of mM per kilo of H_2O. The values for $f = 4.0$ and $K = 1.67$ are assumed.

Intercept = total diffusible Ca (1)	Slope (m) (2)	$[Ca^{++}] = \dfrac{mK}{f-m}$ (3)	$CaCit^- =$ (1) − (3) (4)	Total citrate (5)	$Cit^= =$ (5) − (4) (6)	$K = \dfrac{(3) \times (6)}{(4)}$ (7)	pK_{CaCit^-} (8)
0.7	1.18	0.7	0	0	0		
1.2	1.05	0.595	0.605	0.96	0.355	0.34	3.47
1.4	0.98	0.54	0.86	1.90	1.04	0.65	3.19
2.1	0.85	0.45	1.65	3.82	2.17	0.59	3.23
2.7	0.74	0.38	2.32	5.72	3.40	0.56	3.25
3.2	0.57	0.28	2.92	9.60	6.68	0.64	3.19

and 5.4 mM per kilo of H_2O, respectively. It is seen that the values of the intercepts at the calcium axis become larger with increasing concentrations of citrate (Fig. 6). It is clear that these values must represent calcium

ions and calcium bound to citrate. In the original solution, 13 per cent of the total calcium is in the ionized form. The maximum value for the total diffusible calcium obtained after the addition of 10 mM of sodium citrate per liter represents 59 per cent of the total calcium. The values for m decrease from an original value of 1.18 to 0.57 and represent progressively lowered amounts of calcium bound to casein.

The data in Table VI are derived from a consideration of the mass law relationships for calcium caseinate and calcium citrate. The $[Ca^{++}]$ for the casein solutions is determined in the presence of undissociated calcium citrate with the aid of Equation 10. The values for f and K, determined in pure calcium caseinate solutions, are used. With the aid of the calculated $[Ca^{++}]$, it is possible to determine the pK_{CaCit^-} according to the procedure shown in Table VI. There is a very close agreement between the values in Column 8 and the value of 3.22 for pK_{CaCit^-} found by Hastings, McLean, and associates (17) with the frog heart. In view of the agreement of the results here described with those obtained for solutions of $CaCl_2$ and Na_3Cit with the frog heart method, additional evidence is given for the validity of the latter method.

SUMMARY

1. A study has been made of the partition of calcium and protein in solutions of calcium caseinate after ultracentrifugation. The results have been interpreted by assuming that the diffusible calcium remains evenly distributed through the fluid phase, while the calcium associated with casein is sedimented with the protein.

2. Confirming and extending the results of others, we find that the relationship between calcium and protein, in solution, can be expressed, with a high degree of accuracy, by the law of mass action, in a form which yields the dissociation constant of calcium caseinate. This constant is independent of the concentration of protein or of calcium, and is not affected by diluting solutions of calcium caseinate. It varies with different preparations of casein.

3. Addition of sodium chloride to solutions containing calcium and casein increases the dissociation constant, presumably as a function of ionic strength. This relationship has not been studied quantitatively.

4. The calcium-combining power of casein, at $[Ca^{++}] = \infty$, has been determined by extrapolation from data over a wide range of $[Ca^{++}]$. For the samples of casein examined the factor for converting gm. of protein to mM, in terms of maximum calcium-combining power, was found to be 0.40.

5. Contrary to the findings of many investigators, neither the calcium-combining power of casein nor the dissociation constant of calcium caseinate

is affected by changes in hydrogen ion concentration within a range of pH 6.3 to 8.5. This is in accord with the hypothesis that calcium combines with the zwitter ion $COO^- \cdot R \cdot NH_3^+$ as well as with the protein ion $COO^- \cdot R \cdot NH_2$. Below pH 6.3 a change in the equilibrium occurs, interpreted as a reduction in the value of $pK_{CaCasein}$. Above pH 8.5 the relationships are no longer linear.

6. When citrate is added to solutions containing calcium and casein, the diffusible calcium increases and $[Ca^{++}]$ decreases, as predicted from the dissociation constant of calcium citrate. This is additional evidence for the reliability of the methods employed, and for the applicability of the law of mass action to the relationships between calcium, protein, and citrate.

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STUDIES ON THE CALCIUM-PROTEIN RELATIONSHIP WITH THE AID OF THE ULTRACENTRIFUGE*

II. OBSERVATIONS ON SERUM

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(From the Biochemical Laboratory, University of Virginia, University)

In Paper I, calcium and casein were found to have a quantitative relationship which could be explained by the law of mass action. McLean and Hastings (1) concluded that calcium proteinate of blood serum behaves as a weak electrolyte whose dissociation, as a first approximation, follows the law of mass action. The present communication reports the results of an investigation of the calcium-protein relationship in serum, with the aid of the ultracentrifuge.

Methods

The ultracentrifuge has been described (2, 3). Serum was obtained from the blood of hospitalized patients and from normal horses.[1] Total nitrogen was determined by the micro-Kjeldahl procedure and was converted by the factor 6.3. Albumin and globulin were partitioned with 22 per cent Na_2SO_4. Non-protein nitrogen was determined by the Kjeldahl method or by nesslerization after precipitation of the protein with trichloroacetic acid. Total solids were determined in a large number of original and centrifuged samples of serum after drying for 48 hours at 105°. These results were plotted against protein nitrogen and the equation $98 - 0.56$ N was used subsequently for determining the percentage concentration of water. The pH of the sera was determined in many cases with the glass electrode but no attempt was made to adjust the reaction before centrifuging. Calcium was determined in trichloroacetic acid filtrates as described by Peters and Van Slyke (4). The symbols used are described in Paper I and are similar to those used by McLean and Hastings (1).

Distribution of Calcium and Protein of Serum after Centrifugation—The concentrations of protein and calcium in nine equally divided fractions of an ultracentrifuged human serum are shown in Fig. 1. The ultracentrifuge provides a method for obtaining a series of solutions from a single serum for a study of the distribution of calcium and protein.

Relationship of Calcium and Protein in Serum—A graphic presentation of the calcium and protein distribution of six centrifuged sera obtained from hypertensive patients is shown in Fig. 2.   The graphs obtained consist of at least two substantially linear segments whose points of intersection vary in respect to the protein concentration.   The broken lines serve to indicate the extent to which segments of the graphs differ in slope.

The individual and mean concentrations for diffusible calcium are indicated in Fig. 2.   The assumption is made that the intercept on the Ca axis expresses the values for ionized plus diffusible non-ionized calcium. These values vary from 1.6 to 1.9 mM of calcium per kilo of H_2O, with a mean
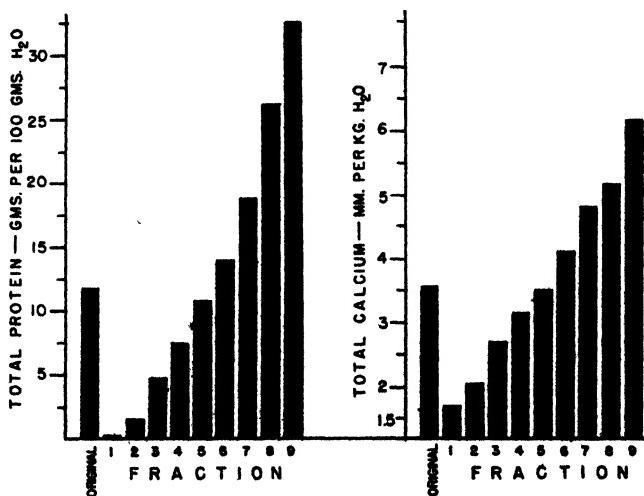
Fig. 1. Distribution of protein and calcium in nine fractions of an ultracentrifuged serum.

value of 1.75.   This range is considerably higher than calcium ion concentrations determined by McLean and Hastings (1) who found the extremes to be 1.10 to 1.40 mM with a mean of 1.25 mM.   The difference of 0.5 mM in the mean values of calcium as determined by the ultracentrifuge and by the frog heart technique may be found to represent non-ionizable diffusible calcium.

On the basis of their calculations and determinations, McLean and Hastings used a nomogram for estimating the Ca ion from total calcium and total protein.   The [Ca^{++}] for six samples of serum (Fig. 2) was calculated by means of the McLean-Hastings nomogram and compared with the total diffusible calcium (Table I).   The discrepancies in Columns 3 and 4 would indicate that appreciable amounts of non-ionized diffusible
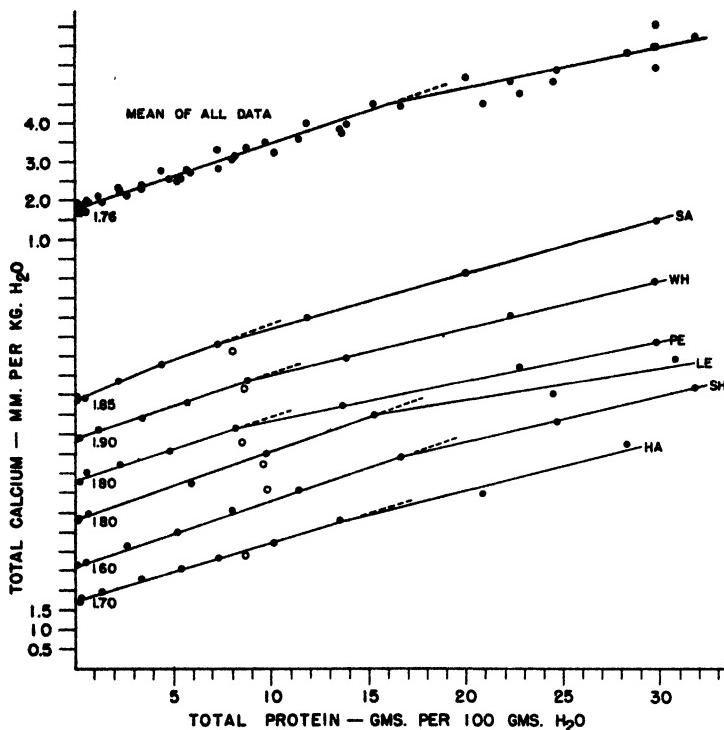
FIG. 2. Serum of six hypertensive patients (represented by the initials HA, etc.) centrifuged for 4½ hours at 1000 revolutions per second. The broken lines indicate the extent to which segments differ in slope. The ● represents centrifuged fractions and ○ represents the original sera.

TABLE I

Comparison of [Ca⁺⁺] in Serum Computed from McLean-Hastings Nomogram and Total Diffusible Calcium Determined with Ultracentrifuge

Patient	Total protein	Total Ca	Ca⁺⁺ from McLean-Hastings nomogram	Total diffusible Ca (ultra-centrifuge)	Difference (4) − (3)
	(1)	(2)	(3)	(4)	
	gm. per 100 gm. H₂O	mM per kg. H₂O	mM per kg. H₂O	mM per kg. H₂O	mM per kg. H₂O
HA	7.90	2.88	1.16	1.70	0.54
SH	8.50	3.12	1.42	1.60	0.18
LE	8.70	3.22	1.25	1.80	0.55
PE	7.75	2.76	1.13	1.80	0.67
WH	7.85	3.14	1.29	1.90	0.61
SA	7.35	3.12	1.36	1.85	0.49

calcium are present in these sera. Similar discrepancies were noted in another group of eighteen sera. Since the McLean-Hastings formulation does not take the relatively large amount of non-ionized calcium into consideration, it is obvious that calculation of the ionization constant of calcium proteinate (pK_{CaProt}) by this method cannot always be correct.

The regression equation for the mean of the data in Fig. 2 is given by [total Ca] = 0.15 Prot + 1.76. In a series of eighteen cases, which comprised a variety of diseases, the sera were centrifuged with a less efficient rotor and the data were plotted. The range of the calcium and protein concentrations was not marked and it was necessary to extrapolate an appreciable portion of the curve. In this set the mean regression equation was [total Ca] = 0.17 Prot + 1.5. The mean regression equation compiled from the data in the literature by McLean and Hasting is [total Ca] = 0.188 Prot + 1.4.

Those equations obtained by collection of data for calcium and protein in the sera of a large number of patients are rough approximations of the true values, since there are wide variations and it is impossible to obtain samples which could be plotted close to the calcium axis. It follows that the ionization constants for calcium proteinate obtained by this method are probably not correct.

The original values for calcium and protein do not fall on the lines of the plots (Fig. 2) obtained from the centrifuged fractions. An explanation for these deviations from the respective graphs is not clear. At present the abrupt changes in slopes cannot be correlated with any other known factors. There appears to be an apparent linear relationship between calcium and protein within the physiological range, which is represented by the initial portion of the curve.

Albumin-Globulin Ratio and Calcium-Protein Relationship—The albumin-globulin ratios and the graphs for the calcium-protein relationship in the ultracentrifuged fractions of four sera are shown in Fig. 3. The spread of the protein values is not particularly marked because the rotor used was an earlier, less efficient model. It is generally recognized that the albumin-globulin ratio obtained by salting-out procedures is not a true indication for the distribution of serum proteins, particularly as related to the globulins. The albumin-globulin ratios determined for the respective fractions show marked variations, because the absolute amount of protein is changed and the heavier globulin molecules sediment more rapidly. These sera are typical of the many studied in having individual distribution patterns for albumin and globulin. The distribution of the proteins as shown in Fig. 3 yields little information on the inclination of the line or the discontinuity of the slope. This study would probably be more fruitful if the distribution of protein fractions were made by the electrophoretic procedure.
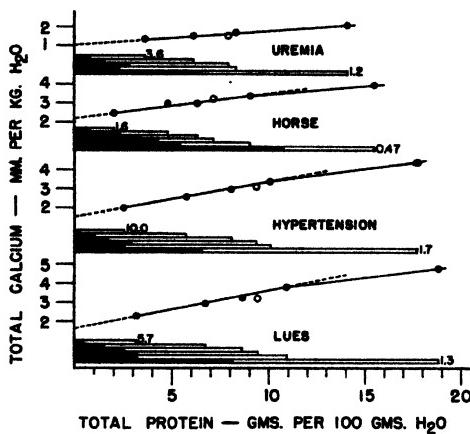
FIG. 3. Graphs showing the albumin-globulin concentrations and values of fractionated serum. The solid boxes represent globulin; the clear ones, albumin. The symbols have the same significance as in Fig. 2.
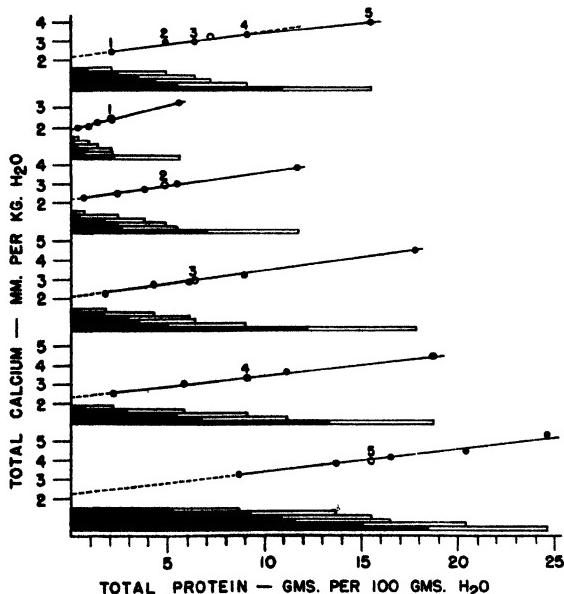
FIG. 4. Albumin-globulin fractions obtained after recentrifugation of serum. The figures on the curves represent fraction numbers. The solid boxes represent globulin; the clear boxes, albumin. The symbols have the same significance as in Fig. 2.

Studies on Recentrifuged Fractions—Normal horse serum was centrifuged and divided into five fractions; each of these fractions was centrifuged and again fractionated into five portions. The data for the centrifuged original serum and its fractions are presented in Fig. 4.

The points of intercept with the calcium axis for the original and the five fractions are 2.2, 1.9, 2.2, 2.1, 2.3, and 2.2 mM of calcium per kilo of H_2O, respectively. This would indicate that the diffusible calcium is uniformly distributed and is not affected by the protein concentration. The values for all the slopes with the exception of Fraction 1 are fairly constant.
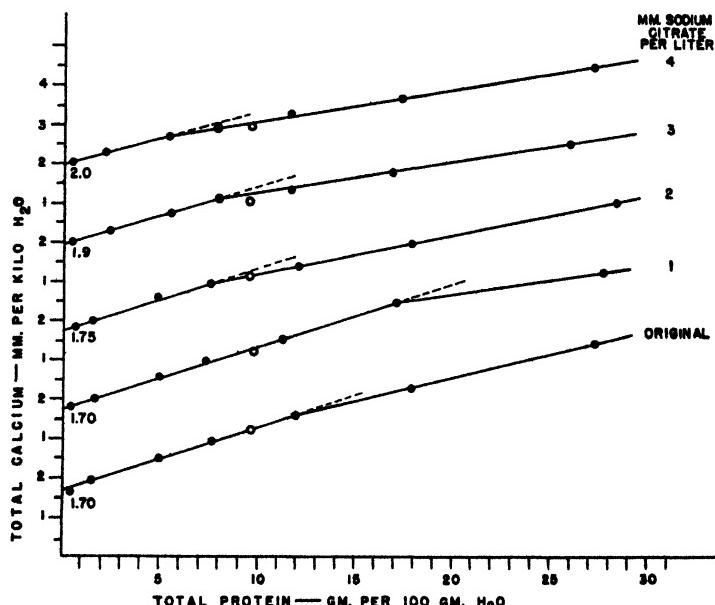
Fig. 5. Effect of addition of 1, 2, 3, and 4 mM of sodium citrate to the centrifuged fractions of horse serum. The symbols have the same significance as in Fig. 2.

There is a definite discontinuity in the slope only in the original serum. To be certain that small differences in the values of the intercepts were not real, another sample of horse serum was centrifuged and the fractions recentrifuged. The results of this experiment were as follows: original serum and Fractions 1 to 4 had values of 2.5, 2.4, 2.5, 2.5, and 2.4 mM of calcium per kilo of H_2O, respectively. These experiments appear to confirm the assumption that the intercept at the calcium axis represents diffusible calcium which remains constant in all fractions. It is difficult to correlate the data in this figure with the albumin-globulin ratios or with the respective protein fractions.

Effect of Sodium Citrate on Diffusible Calcium—The addition to human serum of sodium citrate in concentrations of 1, 2, 3, and 4 mM per liter causes a slight but definite increase in the concentration of diffusible calcium (Fig. 5). A similar experiment with horse serum showed this same result. The position of the points at which there is a discontinuity in the slope is influenced by the amount of citrate added. It is probable that further additions of sodium citrate would cause appreciable increases in the diffusible calcium. According to Shelling and Maslow (5), the addition of extremely large amounts of citrate to serum causes nearly 100 per cent of the calcium to become ultrafiltrable.

Effect of pH upon Diffusible Calcium in Serum—Aliquots of a single sample of human serum were adjusted to varying hydrogen ion concentrations with small amounts of NaOH and HCl and were centrifuged for 7

TABLE II

Effect of pH on Diffusible Calcium of Serum

pH	Serum 1. Diffusible Ca		pH	Serum 2. Diffusible Ca	
	mM per kg. H₂O	*per cent of total*		*mM per kg. H₂O*	*per cent of total*
7.0	1.75	51.4	6.1	1.60	54.0
7.1	1.78	52.2	6.6	1.58	53.5
7.2	1.75	51.4	7.0	1.58	53.5
7.3	1.60	47.0	7.3	1.58	53.5
7.4	1.62	47.6	7.5	1.42	48.0
7.6	1.52	44.6	7.7	1.30	44.0
7.8	1.52	44.6	7.85	1.23	41.5
			8.1	1.23	41.5

hours at 1000 revolutions per second. The top fractions of these sera were free or contained only a slight trace of protein (tested with trichloroacetic acid) and their values for calcium were taken to represent diffusible calcium.

The results of two typical experiments are shown in Table II. There is a definite decrease in the diffusible calcium with increased pH. It is important to note that these changes are not uniform but proceed in an irregular stepwise manner. Furthermore, there is no definite pH at which one can predict a sudden change in the concentration of diffusible calcium in different sera. Similar changes were noted but not commented upon by Smith (6) in ultrafiltrates of serum. The changes noted in diffusible calcium of serum at different hydrogen ion concentrations confirm the findings of other investigators (6–8). The frequent stepwise changes noted for serum with small differences in pH stand out in contrast to the constancy in diffusible calcium over a wide range of hydrogen ion concentration in calcium caseinate solutions.

In solutions of a single protein (2), calcium and casein bear a relationship to each another which follows the law of mass action. On the other hand, the data for blood serum obtained in these experiments cannot be explained satisfactorily by a single assumption of a protein-calcium equilibrium. This does not deny the validity of the mass law for the calcium-protein relationship but stresses the inability to evaluate properly data obtained in a complex system.

On the basis of their findings, McLean and Hastings presented a formulation from which the calcium ion concentration of serum could be calculated from total protein and total calcium. They assumed that practically all the diffusible calcium was in the ionized state, a single pK $_{CaProt}$, and a constant albumin-globulin ratio. However, they pointed out the possibility of the presence of small amounts of citrate-like materials in serum. A comparison of the total diffusible Ca concentration obtained with the ultracentrifuge and the calcium ion concentration calculated with the McLean-Hastings nomogram showed differences between 10 and 40 per cent. These discrepancies may be explained by the work of Drinker, Green, and Hastings (9) who found that the dissociation constants of the different globulin fractions of serum are different and by the observation that the proteins of normal and pathological sera varied greatly (10, 11). In addition, the factors affecting the concentration of citrate-like materials in serum are not fully appreciated.

Calcium-combining powers of serum proteins have been presented by numerous workers (1, 9, 12) and in each case these values differ. Even under the best circumstances, it is improbable that the properties are identical with those occurring in the native state. Therefore, values for calcium-combining properties are probably rough approximations at best.

It has been shown in this work that there are marked variations in the albumin and globulin concentrations of the respective centrifuged fractions of a single serum. Despite these variations, the calcium and protein concentrations exhibit a substantially linear relationship over a limited range. If it is assumed that the serum proteins exert their respective calcium-combining properties, it would be impossible to obtain the relationship observed. It must follow, therefore, that albumin and globulins in serum react substantially alike to calcium, as a first approximation, over a limited range of calcium and protein concentrations.

No explanation could be found for the discontinuities in the slopes when the protein concentration is increased. An exception to this is seen in the graphs for recentrifuged fractions shown in Fig. 4. Although there are marked variations in the concentration of protein and in the distribution of albumin and globulin, the slopes appear to be linear.

SUMMARY

The relation of total calcium to total protein in horse and pathological human sera was studied with the aid of the ultracentrifuge. Discontinuities in the slopes of graphs representing the calcium and protein concentrations were observed in the majority of the centrifuged sera.

Evidence is presented to show that the total diffusible calcium remains constant in all centrifuged fractions of a single serum.

A study has been made of the McLean-Hastings procedure of applying a single mass law equation to the total calcium and total protein concentrations of serum for the determination of the calcium ion concentration. This method does not appear to be reliable and the possible reasons for this are discussed.

Sodium citrate increased the diffusible calcium and decreased the calcium bound to protein.

A gradual rise in pH caused an irregular fall in the concentration of diffusible calcium.

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STUDIES ON THE CALCIUM-PROTEIN RELATIONSHIP WITH THE AID OF THE ULTRACENTRIFUGE*

III. INFLUENCE OF AUGMENTATION OF SERUM WITH CALCIUM AND PHOSPHATE SALTS

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Since the calcium-protein relationship for a purified protein is adequately described by the law of mass action (1), it follows that the inconsistencies between the total calcium and total protein for sera (2) probably represent the cumulative effect of a number of variables. The present report deals with some of these variables as follows: the effect of excessive amounts of CaCl₂ on the calcium-protein relationship, and the effect of excessive amounts of calcium and phosphorus on the calcium-phosphorus-protein relationship in serum.

There is evidence in the literature to show that the addition of excessive amounts of calcium or phosphorus to serum results in the formation of compounds of a colloidal nature whose exact composition is unknown. Such complexes must affect the concentration of ionized calcium and therefore the calcium-protein relationship.

The ultracentrifuge and the methods for determining nitrogen and calcium have been described (1). Phosphorus was determined on trichloroacetic acid filtrates by the Fiske and Subbarow method (3) as adapted to the photoelectric colorimeter.[1] The pH was adjusted to the original value of the serum (about 7.8) after either Na₂HPO₄ or CaCl₂ was added. The pellet that usually formed after the addition of salts was not analyzed, thus representing a loss of protein, calcium, or phosphorus.

Calcium and Protein Distribution in Serum after Augmentation with Calcium—Varying amounts of CaCl₂ were added to aliquots of stock human and horse serum, respectively. Graphs showing the calcium-protein relationships for the respective sera are presented in Figs. 1 and 2. The results are plotted as broken line graphs, since no linear relationship could be established. The general slope of the lines increases with the increased calcium concentration in a manner noted for calcium-casein solutions (1). The graphs obtained represent the combined effects of at least three

[1] Evelyn photoelectric colorimeter, Notes on operation, Rubicon Company, Philadelphia (1939).

factors; namely, the distribution of the individual serum proteins, the differences in their respective dissociation constants, and the sedimentation of calcium compounds not associated with protein.

Greenberg and Larson (4) determined the dissociation constant of cal-
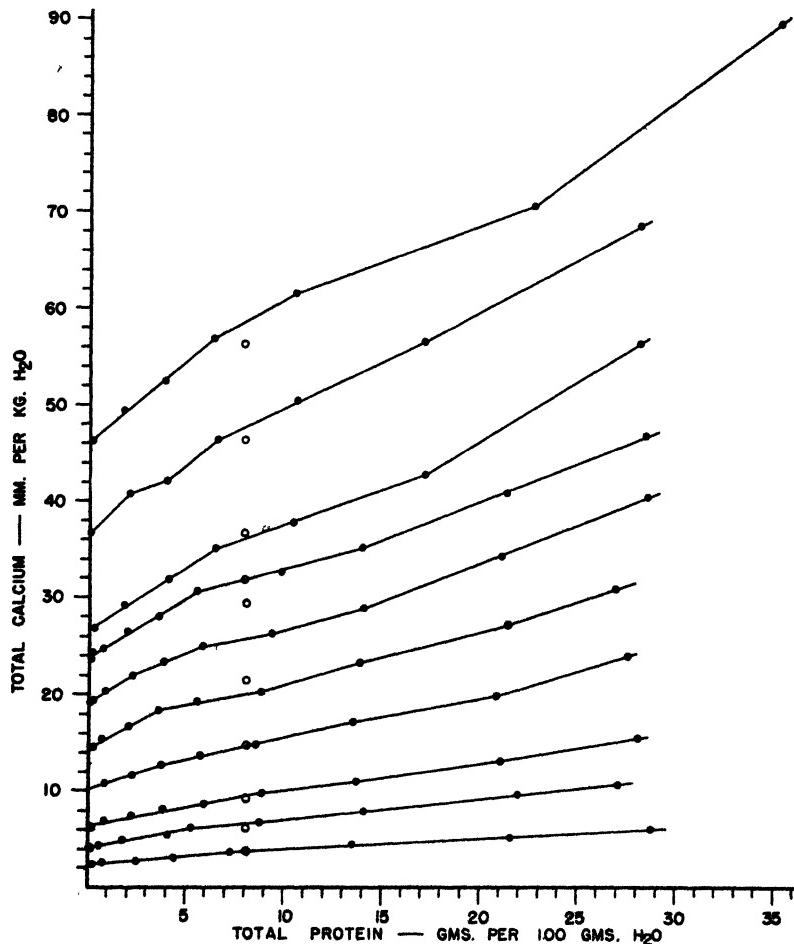
FIG. 1. A graph of the calcium and protein concentrations of a single sample of horse serum augmented with varying quantities of $CaCl_2$. The ● represents values for the centrifuged fractions and the ○ for the original serum.

cium proteinate and the maximal calcium-combining power of protein by assuming that the law of mass action was applicable to the calcium and protein of serum. These workers combined the data for beef, dog, and human sera for obtaining their results. To test the validity of the

Greenberg-Larson procedure for serum, the data from Figs. 1 and 2 were used. If the intercept is assumed to represent the [Ca^{++}], the amount of bound calcium is readily determined from the total calcium value of the original serum. Plotting the reciprocal of calcium bound per 10 gm. of protein and the calcium ion concentration for human and horse serum yielded results from which the mathematical data could not be interpreted. It seems logical to assume that the application of the law of mass action to serum would require the accurate analysis of the [Ca^{++}], the un-ionized diffusible calcium, the distribution of the different proteins, and the individual constants for the respective proteins.
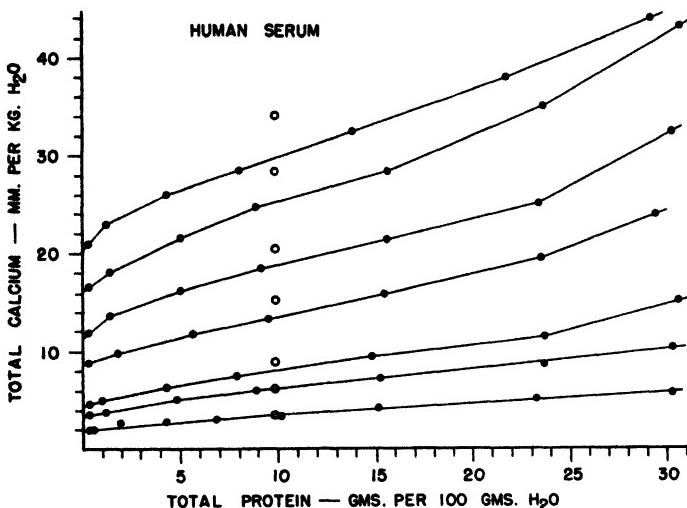
FIG. 2. A graph of the calcium and protein concentrations of a single sample of human serum augmented with varying quantities of CaCl$_2$. The symbols have the same significance as in Fig. 1.

Calcium-Phosphate-Protein Equilibrium under Varying Conditions—The condition of the inorganic phosphorus in normal blood has been discussed by Grollman (5). The relationship of calcium and phosphorus in serum has been reviewed by Schmidt and Greenberg (6) and McLean and Hinrichs (7). There appears to be no agreement concerning the amount and the composition of a colloidal calcium-phosphate complex in serum. It is generally agreed that the excessive quantities of calcium or phosphate salts influence the ionization of phosphate or calcium by the formation of a colloidal complex.

Normal Serum—Analysis of fractionated centrifuged normal horse serum showed that the inorganic phosphorus concentration increases

progressively with protein concentration. Differences of 0.14 and 0.15 mM of P per kilo of H_2O were obtained between the top and bottom fractions of two sera. In order to obtain more detailed data, horse serum was centrifuged and divided into five fractions; each fraction was again separated into five portions after centrifugation. The data for calcium, phosphorus, and protein are shown in Fig. 3. The relationship between calcium and protein is irregular in comparison with the phosphorus-protein distribution. The spread of calcium values in this graph is probably an expression of the marked differences in the protein distribution of the recentrifuged fractions. A linear relationship for phosphorus and protein is seen for all but the first portion of the plot which represents Fraction 1 of the recentrifuged serum.

The results obtained indicate that phosphorus is present in normal horse
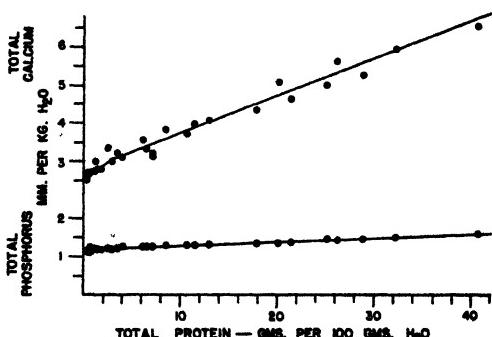
FIG. 3. The distribution of calcium, phosphorus, and protein in recentrifuged fractions of horse serum.

serum not only as a simple diffusible phosphate but also in combination with protein. In contrast to the discrepant relation of calcium to protein, phosphorus varies only with the total protein concentration. Since analyses were carried out on trichloroacetic acid filtrates, it is probable that phosphate forms a loose combination with protein.

Addition of Calcium Chloride to Serum—Calcium chloride was added to one sample of serum in varying quantities to bring the calcium concentration as high as 80 mM per kilo of H_2O. These sera were centrifuged for 2½ hours and fractionated as previously described.

The results for the calcium-protein relationship as shown in Fig. 4 are similar to those discussed in previous experiments. The rate of fall of diffusible phosphate concentration (intercept at phosphorus axis) is dependent on the concentration of calcium present. This relationship is shown in Fig. 5 where it can be seen that the diffusible P decreases markedly with small additions of calcium. Despite the addition of extremely

large amounts of calcium, diffusible P does not disappear but remains present in small traces. Grollman (5) noted a similar phenomenon in
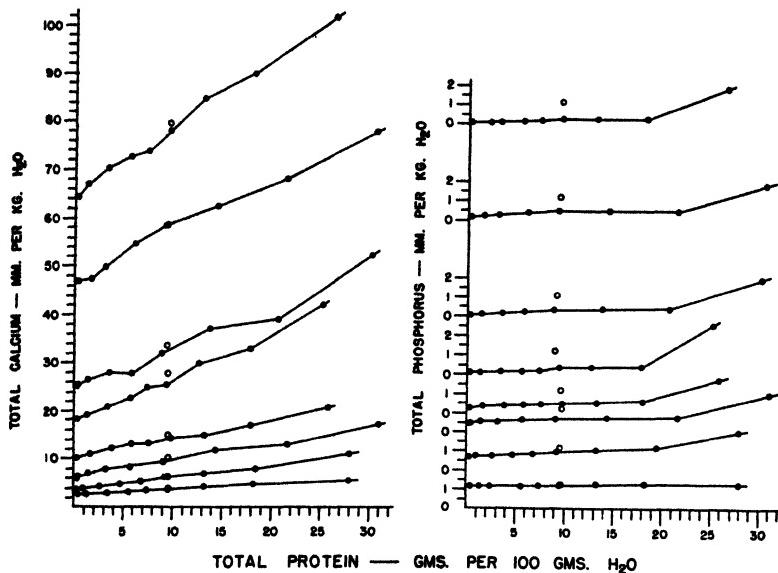
FIG. 4. Graphs showing the effect of augmenting a single sample of horse serum with CaCl$_2$ on the calcium-protein and the phosphorus-protein relationship. The symbols have the same significance as in Fig. 1.
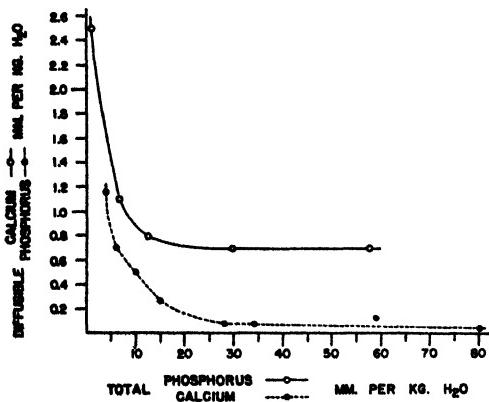
FIG. 5. The calcium-phosphorus relationships after augmenting horse serum with CaCl$_2$ or Na$_2$HPO$_4$.

ultrafiltration studies. These results would appear to be due to a reaction between calcium and phosphorus with the formation of a large molecule,

presumably colloidal in nature. The marked increase in the P concentration in the last fraction would tend to confirm this idea.

Addition of Phosphate to Serum—Disodium hydrogen phosphate was added to one sample of serum in varying quantities to bring the P concentration as high as 57.5 mM per kilo of H_2O. These sera were centrifuged for $2\frac{1}{2}$ hours and the fractions analyzed for calcium, phosphorus, and protein.
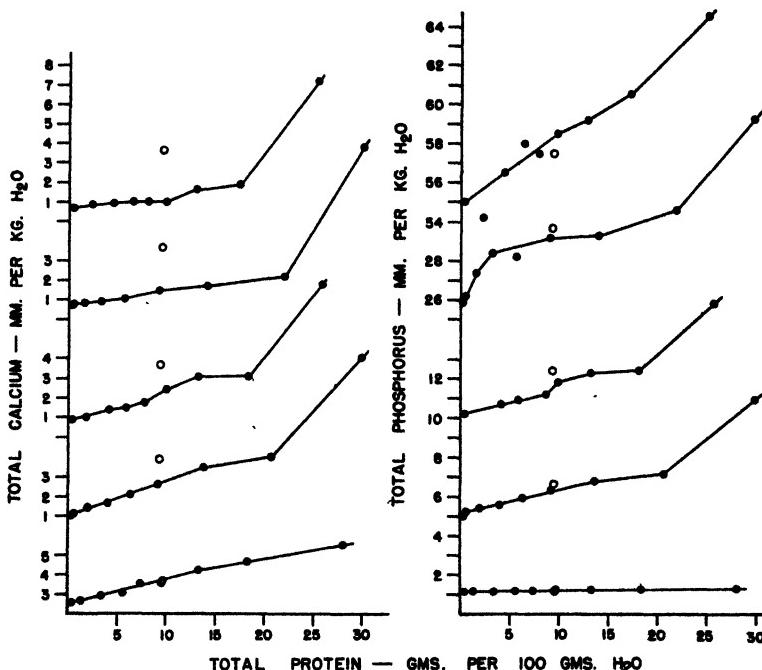
FIG. 6. Graphs showing effect of augmenting a single sample of horse serum with Na_2HPO_4 on the calcium-protein and the phosphorus-protein relationship. The symbols have the same significance as in Fig. 1.

Figs. 5 and 6 show the significant changes for calcium and phosphorus. Diffusible calcium concentrations decrease sharply after the addition of small amounts of phosphate but tend to remain constant after the addition of larger amounts. The marked increase of calcium in the last fractions appears to confirm the idea that a calcium-phosphate complex is formed under these experimental conditions. It is obvious that calcium is withdrawn from both the diffusible and protein-bound calcium fractions of serum.

If the intercept at the P axis is interpreted as diffusible phosphorus, it

is seen that the amount of bound phosphorus increases with the concentration of phosphate. The sudden rise in total P values of the last fractions can be attributed to the rapidly sedimenting calcium-phosphate complex.

SUMMARY

A study has been made of the effects of adding calcium and phosphate salts to serum on the calcium-protein, phosphorus-protein, and calcium-phosphate relationships with the aid of the ultracentrifuge.

Augmentation of serum with calcium shows that there is a poor correlation between protein and calcium. This would indicate that the equilibrium between serum proteins and calcium is too complex to be interpreted by a single mass law relationship.

Evidence is presented to show that a phosphate-protein compound normally occurs in horse serum.

A rapidly sedimenting molecule containing calcium and phosphorus is formed *in vitro* when either calcium or phosphate salts are added to serum. This calcium-phosphate complex does not appear to be directly associated with protein.

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STUDIES ON BILE ACID METABOLISM

I. THE FATE OF CHOLIC ACID IN THE GUINEA PIG

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It is well known that bile acids are constantly being synthesized by the normal animal. Since these acids are not excreted in amounts comparable to those formed (1) and normally do not accumulate in the animal body, it is apparent that these substances must be catabolized. At present, however, little is known of either the site, mechanism, or products of bile acid decomposition.

The problem of bile acid catabolism has been approached in our laboratory through a study of the fate of cholic acid in the guinea pig. This study has shown that cholic acid injected intravenously disappears from the body and is not eliminated as such in the excreta. The disappearance of this bile acid is due largely, if not entirely, to decomposition within the cecum through the action of bacteria. Details of these findings are reported here.

Methods

Method of Determining Cholic Acid—The method used for determining cholic acid was based on a modification (2) of the Gregory-Pascoe (3, 4) procedure. Analyses were carried out on alcoholic extracts of the various organs, excreta, body fluids, and bacteriological media. An aliquot of an extract, estimated to contain from 0.15 to 0.8 mg. of cholic acid, was evaporated to dryness on the steam bath. The residue was dissolved in 2.5 cc. of 60 per cent acetic acid; 1 cc. portions of the resulting solution were pipetted into each of two small test-tubes, to which 6 cc. of 16 N sulfuric acid were added. 1 cc. of a 0.9 per cent solution of *freshly distilled* furfural in 60 per cent acetic acid was added to one of these tubes; to the other tube, which served as a blank, 1 cc. of 60 per cent acetic acid was added. The contents of these tubes were mixed thoroughly, heated in a water bath at 67° for 12 minutes, and then cooled to room temperature. The colors of the resulting solutions were compared in a colorimeter with that of a suitable cholic acid standard, with red light (6100 to 6900 Å.) for illumination. Cholic acid standards were prepared as follows: 5 cc. portions of two stock alcoholic solutions, containing 0.25 and 0.5 mg. of cholic acid respectively, were evaporated to dryness and treated exactly as were the unknowns.

The cholic acid content of the unknown was calculated by deducting the color equivalent of the blank tube from that of the tube containing furfural.   Correction for the blank was unnecessary when extracts of blood plasma, bile, and media were analyzed.   It was necessary in the analysis of extracts of the other materials studied, since these extracts contained non-specific substances which gave a small but measurable absorption of light (6100 to 6900 Å.) when heated with sulfuric acid alone.

Preparation of Extracts—For blood plasma, urine, fistula bile, and bacteriological media, 2 cc. quantities of the respective fluids were added dropwise to approximately 40 cc. portions of absolute alcohol; the mixtures were heated to boiling, filtered, cooled, and diluted to 50 cc. volume.

For liver the excised organ was weighed and cut into small slices.   A 5 gm. portion was ground with sand in a mortar and extracted with four successive 25 cc. portions of boiling absolute alcohol.   The extracts were combined, filtered, cooled, and made up to 100 cc. volume.

Feces were collected at 24 hour periods, dehydrated with alcohol, powdered in a mortar, and extracted with one 100 cc. portion followed by two 40 cc. portions of boiling alcohol.   The combined extracts were filtered, cooled, and made up to 200 cc. volume.

In the case of the stomach, small and large intestine, cecum, and gallbladder, and the contents of these organs, the intact organ, including contents, was placed in a beaker, opened longitudinally, and cut into fine pieces.   The entire contents of the vessel were then extracted with boiling alcohol, the same procedure being used as for feces.

EXPERIMENTAL

Presence of Cholic Acid in Normal Guinea Pigs—Before a study of the fate of cholic acid in the guinea pig was made, it was necessary to determine whether organs of the normal animal contained this bile acid.   Accordingly, animals weighing 500 to 700 gm. were placed in metabolism cages and urine and feces were collected for a 24 hour period.   The guinea pigs were then sacrificed and the various organs, body fluids, and excreta analyzed for cholic acid, according to the procedure described above.

The results of these analyses, as recorded in Table I, show that in the normal guinea pig the various body fluids, excreta, and gastrointestinal organs and contents contained materials which gave a color equivalent to approximately 41 mg. of cholic acid.   It was questionable, however, whether cholic acid was actually present in the normal animal except for traces in bile, since in the analysis of all organs, etc., other than gallbladder and contents, a brown color was obtained instead of the blue characteristic of cholic acid.   Actually this question was immaterial to our study, since the amounts of color-producing substances found in

corresponding organs of different animals were fairly constant, as shown in Table I.

For the present purposes, it has been assumed that the "apparent" cholic acid values represented substances other than cholic acid which react with sulfuric acid and furfural to form pigment. *All subsequent data obtained on animals receiving cholic acid have been corrected with respect to these "blank" values recorded in Table I.*

Recovery of Cholic Acid Added to Organs, Body Fluids, and Excreta of Normal Animals—Experiments were carried out to determine whether cholic acid could be recovered quantitatively when added to the various organs, body fluids, and excreta of the guinea pig. Measured quantities

TABLE I

"Apparent" Cholic Acid Content of Tissues, Body Fluids, and Excreta of Normal Guinea Pigs

Guinea pig No.	"Apparent" cholic acid content								Total in tissues, fluids, and excreta examined
	Blood*	Gall-bladder and bile	Urine†	Feces†	Stomach	Intestine	Cecum	Liver	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.5	1.6	0.5	6.5	4.1	5.5	10.6	5.1	34.4
2	0.9	1.7	0.5	4.5	6.6	7.9	12.0	6.7	40.8
3	0.9	1.7	1.1	6.5	5.3	7.9	11.0	10.8	45.2
4	0.6	2.0	1.3	6.2	4.6	7.6	11.1	10.4	43.8
5	0.5	0.7	0.7	6.6	7.2	8.3	7 9	8.6	40.5
Average. .	0.7	1.5	0.8	6.1	5.6	7.4	10.5	8.3	40.9

* "Apparent" cholic acid content of blood was calculated on the assumption that blood amounts to 8 per cent of the total weight of a guinea pig, and that equal amounts of cholic acid were present in red blood cells and plasma.

† 24 hour collections of urine and feces.

of sodium cholate were added to blood plasma, bile, urine, feces, finely ground liver, and to the stomach, small intestine, and cecum, and their contents. These materials were extracted with alcohol and the extracts analyzed for cholic acid.

When 0.1 to 0.2 mg. quantities of cholic acid were added to 1 cc. portions of either blood plasma or bile, the recovery was 97 ± 2 per cent. When similar amounts of cholic acid were added to 1 cc. of urine, the recovery was 94 ± 3 per cent. When 10 mg. of cholic acid were added to 5 gm. portions of liver, to 24 hour collections of feces, or to the entire stomach, small intestine, or cecum, the recoveries for the respective materials were as follows: liver 88 ± 4 per cent, feces 85 ± 4 per cent, stomach and contents 86 ± 4 per cent, small intestine and contents 90 ± 4 per cent,

cecum and contents 93 ± 3 per cent.   Although theoretical recovery was possible only with blood plasma and bile, the results obtained with other organs were deemed adequate for present purposes.

Experiment 1.   Elimination of Cholic Acid in Bile Following Intravenous Injection—This experiment was carried out as a guide to further studies of the disposition of cholic acid in the guinea pig.   Biliary fistulae of the Rous-McMaster type (5) were prepared in a group of guinea pigs.   Immediately thereafter measured quantities of a 4 per cent solution of sodium cholate in physiological saline were injected into the jugular veins of some of these animals.   Similar quantities of saline were injected into other

TABLE II

Experiment 1. Elimination of Cholic Acid in Bile Following Intravenous Injection

Guinea pig No.	Cholic acid injected	Cholic acid recovered			Total cholic acid recovered	Recovery of injected cholic acid
		0–1 hr.	1–2 hrs.	2–6 hrs		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	161	103.4	34.5	24.5	162.4	100.9
2	183	137.5	19.7	21.7	178.9	97.7
3	92	69.7	13.5	9.4	92.6	100.6
4	84	57.3	17.6	10.1	85.0	101.2
5	185	105.3	33.1	44.2	182.6	98.7
6	170	27.0	17.9	124.0	168.9	99.3
7	92	61.0	22.1	11.7	94.8	103.0
8	80	50.9	18.4	13.4	82.7	103.3
Average	...					100.6
				(0–6 hrs.)		
9	Control			3.9		
10	"			5.1		
11	"			5.1		

control animals.   The preparation of the fistulae and the injections of sodium cholate and saline solutions were carried out under nembutal and light ether anesthesia.   Bile was collected at various intervals after the injections, extracted, and analyzed for cholic acid.

The results of this experiment, summarized in Table II, show that cholic acid, administered intravenously, was rapidly secreted into the bile, just as in animals that normally produce large quantities of this bile acid.   Within 1 hour after administration, 50 per cent of the cholic acid had been recovered; within 6 hours, recovery was complete.

Experiment 2.   Amounts of Cholic Acid Found in Gastrointestinal Tract, Liver, Blood, and Excreta at Various Periods after Injection—Measured

quantities of a 4 per cent solution of sodium cholate in physiological saline (the equivalent of approximately 0.25 gm. of cholic acid per kilo of body weight) were injected into the jugular veins of adult guinea pigs. Groups of animals were sacrificed 2, 6, 12, 24, and 96 hours after injection. Liver, blood plasma, urine, feces, and the organs and contents of the gastrointestinal tract were extracted with alcohol and the extracts analyzed for cholic acid. From these results, the proportions of injected cholic acid found in the various organs were calculated. These latter data are summarized in Table III.

As the data in Table III show, there was a progressive loss of cholic acid from the various organs and fluids examined. Whereas 2 hours after injection the average recovery of cholic acid amounted to 66 per cent, only 45, 33, and 22 per cent could be recovered at the end of 6, 12, and 24 hours, respectively. After 96 hours, less than 5 per cent of the injected material could be recovered.

The distribution and rate of disappearance of cholic acid in the various organs were of interest. Although there were considerable variations between individual animals, a definite trend was discernible. Cholic acid was removed from the circulation very rapidly, for at the end of 2 hours less than 4 per cent of the injected material remained in the blood of any animal. In spite of this rapid removal, traces of cholic acid were still present in the blood at the end of 24 hours.

Approximately 60 per cent of the bile acid injected was found in the gallbladder and gastrointestinal tract at the end of 2 hours, the gallbladder containing 16 per cent, the stomach 5 per cent, the small intestine 18 per cent, and the cecum 22 per cent. At 6, 12, and 24 hours, the amount of cholic acid in the gallbladder was essentially the same as at 2 hours. The amount in the small intestine decreased rapidly and at 12 hours very little was present. The amount of cholic acid in the stomach was greater at 6 than at 2 hours, probably because of regurgitation of intestinal contents; thereafter, the quantity decreased rapidly. The observations on the cecum were of particular interest. Although there was a progressive decrease in the cholic acid content of this organ, the decline was notably slower than in the stomach and small intestine. Even at 96 hours, the cecum contained detectable amounts of cholic acid.

Little cholic acid was found in the liver at any time. This seemed remarkable, in view of the passage of cholic acid from the blood through the liver into the bile. It was especially noteworthy that cholic acid was not found in either the large intestine, feces, or urine, indicating that cholic acid was not excreted as such by the guinea pig.

Experiment 3. Decomposition of Cholic Acid by Liver Tissue in Vitro—
In view of earlier suggestions as to the rôle of the liver in bile acid catabo-

TABLE III

Experiment 2. Amounts of Cholic Acid Found in Gastrointestinal Tract, Liver, Blood, and Excreta at Various Periods after Injection

Guinea pig No.	Time sacrificed after injection	Cholic acid injected	Per cent of injected cholic acid found*						Per cent recovery of injected cholic acid
			Blood	Gall-bladder and bile	Liver	Small intestine	Stomach	Cecum	
	hrs.	*mg.*							
1	2	152	2.1	16.5	0.2	26.3	12.4	12.9	70.4
2	2	139	2.5	16.2	3.0	21.6	3.4	19.3	66.0
3′	2	128	3.5	10.7	0.0	17.2	2.2	28.0	61.6
4	2	132	3.3	21.4	0.0	7.4	3.2	30.7	66.0
Average			2.9	16.2	0.8	18.1	5.3	22.7	66.0
5	6	119	1.3	26.0	0.9	0.0	8.7	6.6	43.5
6	6	128	1.2	20.3	0.9	12.2	7.7	14.4	56.7
7	6	119	1.3	15.2	0.0	0.0	8.8	21.5	46.8
8	6	83	5.3	21.6	2.2	0.0	4.8	8.4	42.3
9	6	106	3.2	13.3	1.9	0.0	11.1	8.0	37.5
Average			2.5	19.3	1.2	2.4	8.2	11.8	45.4
10	12	131	1.8	18.4	0.0	0.7	5.1	6.1	32.1
11	12	169	1.0	26.0	1.0	0.7	4.4	3.4	36.5
12	12	161	1.0	29.7	2.0	0.0	1.8	6.3	40.8
13	12	146	1.1	10.5	2.4	5.3	2.5	6.1	27.9
14	12	157	0.8	4.5	0.0	0.0	1.4	24.3	31.0
15	12	169	1.2	20.2	0.6	0.0*	0.5	6.0	28.5
16	12	149	1.1	23.3	0.0	0.0	0.0	3.2	27.6
17	12	181	0.8	17.5	2.2	0.8	3.0	16.5	40.8
Average			1.1	18.8	1.0	0.9	2.3	9.0	33.1
18	24	140	1.4	16.1	0.0	0.0	0.6	3.3	21.4
19	24	140	1.5	18.7	0.0	0.0	3.7	0.9	24.8
20	24	134	1.5	11.0	0.0	0.0	2.1	2.3	16.9
21	24	120	1.6	21.0	0.0	0.0	0.0	3.3	25.9
Average			1.5	16.7	0.0	0.0	1.6	2.4	22.2
22	96	99	0.0	0.1	0.0	0.0	0.0	1.2	1.3
23	96	121	0.0	0.0	0.0	0.0	0.0	1.1	1.1
24	96	99	0.0	0.0	0.0	0.0	0.0·	4.9	4.9
Average			0.0	0.0	0.0	0.0	0.0	2.4	2.4

* These recovery values have been corrected for the blank values obtained from analysis of corresponding organs of normal animals. Extracts of the large intestine and contents, urine, and feces were also analyzed for cholic acid, but the results of these analyses are not recorded here, since cholic acid was not present in such extracts at any time of the experiment.

lism (6), attempts were made to determine whether liver tissue could decompose cholic acid *in vitro*. Various preparations of fresh guinea pig liver were made under aseptic conditions. In some experiments, thin slices of liver were prepared; in others, the liver was macerated with sand in a sterile mortar; in still other experiments, physiological saline or glycerol extracts were made. Quantities of these preparations, equivalent to 5 to 10 gm. of liver, were placed in sterile, wide mouth bottles, to which were added 0.02 M phosphate buffer solutions (pH 7 to 8) containing 0.05 to 0.2 per cent cholic acid (as sodium cholate). Glucose (0.5 per cent) was added to some of the preparations. These mixtures were incubated at 38° for various periods up to 6 days. At the end of the incubation period, the mixture in the bottle was extracted thoroughly with alcohol, and the cholic acid content of the extract determined. These results were compared with those of controls analyzed prior to incubation.

Regardless of the technique of preparing the liver tissue, the concentration of cholic acid, the pH of the buffer, or the presence of glucose, no evidence for the decomposition of cholic acid by liver tissue was obtained. Because of the negative nature of these results, the complete data are not presented here.

Experiment 4. Distribution of Cholic Acid in Guinea Pig with Functionally Inactive Cecum—The failure of isolated liver tissue to decompose cholic acid suggested that another organ must be involved in the metabolism of this bile acid. The cecum was implicated by the results of Experiment 2 (Table III), cholic acid appearing in this organ in large amounts shortly after injection and disappearing slowly thereafter. In order to determine more definitely the relation of the cecum to the disappearance of cholic acid, the recovery of this bile acid was studied in the guinea pig with a functionally inactive cecum.

The cecum was rendered functionally inactive by double ligation of the ileum just anterior to the ileocecal junction, the necessary operative procedures being carried out under nembutal and light ether anesthesia. Immediately after ligation of the ileum, a measured quantity of a 4 per cent solution of sodium cholate (equivalent to approximately 0.25 gm. of cholic acid per kilo of body weight) was injected into the jugular vein. 6 hours later, the animal was sacrificed. The various organs of the gastrointestinal tract, liver, blood, and urine were extracted, and the extracts analyzed for cholic acid. The results obtained with eight different animals are summarized in Table IV.

As the data show, the recovery of cholic acid from the blood and gastrointestinal tract was almost theoretical in several animals and averaged 88 per cent in the entire group. It was apparent, therefore, that the cecum played a considerable part in the disappearance of cholic acid, for the

average recovery of this bile acid from animals with functionally active ceca was only 45 per cent (*cf.* Table III, 6 hour group).

Experiment 5. Disappearance of Cholic Acid from Isolated Cecum—Direct evidence of the decomposition of cholic acid in the cecum was obtained by studying the fate of this bile acid in the isolated organ. Under aseptic procedures, the distal end of the ileum and the proximal end of the large intestine were doubly ligated and divided between the ligatures. The entire cecum, with attached stumps of ileum and large intestine, was excised and placed in a sterile, wide mouth bottle. The iso-

TABLE IV

Experiment 4. Distribution of Cholic Acid in Guinea Pig with Functionally Inactive Cecum

Animals sacrificed 6 hours after injection.

Guinea pig No.	Cholic acid injected	Per cent of injected cholic acid found*†					Per cent recovery of injected cholic acid
		Blood	Gallbladder and bile	Liver	Small intestine	Stomach	
	mg.						
1	102	4.6	17.4	0	66.0	5.0	93.0
2	91	7.5	0.7	0	54.9	24.0	87.1
3	102	8.4	3.7	0	50.3	17.4	79.8
4	93	4.8	3.3	0	74.7	4.5	87.3
5	107	4.8	17.8	0	45.4	20.7	88.7
6	93	4.8	5.2	0	49.1	24.3	83.4
7	129	3.9	10.9	1.7	60.1	17.7	94.3
8	129	4.7	4.7	1.2	53.5	25.3	89.4
Average.		5.4	8.0	0.3	56.8	17.4	87.9

* These recovery values have been corrected for the blank values obtained from analysis of corresponding organs of normal animals.

† No cholic acid was found in the cecum. Analyses of extracts of urine and large intestine and contents were also carried out; no cholic acid was found in these extracts.

lated organ was then injected with 15 to 20 cc. of a 0.4 per cent solution of sodium cholate in physiological saline, covered with saline, and incubated at 38° for 48 hours. At the end of the incubation period, the cecum was opened longitudinally, the mixture of saline, cecum, and contents was extracted with ethyl alcohol, and the extract analyzed for cholic acid. Control experiments were carried out simultaneously to determine the recovery of cholic acid from the cecum prior to incubation.

The results of these experiments (Table V) show that the recovery of cholic acid from the cecum prior to incubation averaged 93.5 per cent. After 48 hours incubation, the recovery varied from 1 to 38 per cent with

an average of 14.8 per cent. This finding demonstrated that the cecum played an important rôle in the disappearance of cholic acid from the normal guinea pig.

Experiment 6. Decomposition of Cholic Acid in Media Containing Suspension of Cecal Contents—The following experiment was carried out to determine whether cecal contents could decompose cholic acid. A measured quantity of cecal contents was aspirated aseptically and diluted with 20 volumes of saline. Three 500 cc. Erlenmeyer flasks were prepared, each containing approximately 100 mg. of sodium cholate dissolved in 50 cc. of beef infusion broth. To Flask 1, 0.5 cc. of freshly diluted cecal contents was added; to Flask 2 was added a similar quantity of diluted

TABLE V

Experiment 5. Disappearance of Cholic Acid from Isolated Cecum

Guinea pig No.	Cholic acid injected	Incubation of injected cecum	Recovery of cholic acid injected*	Average recovery
	mg.	*hrs.*	*per cent*	*per cent*
1	70.6	None	100	
2	70.6	"	92	
3	70.6	"	92	
4	70.6	"	90	93.5
1	54.5	48	1	
2	54.5	48	27	
3	54.5	48	10	
4	54.5	48	38	
5	73.7	48	18	
6	69.4	48	11	
7	64.7	48	7	
8	64.0	48	6	14.8

* The recovery values have been corrected for the blank values obtained from the analysis of the normal cecum.

cecal contents which had been heated to boiling for 15 minutes; Flask 3 served as a control. These flasks were incubated at 38° for 48 hours, being shaken at 8 hour intervals throughout this period. Samples were withdrawn from each flask before and after incubation, extracted, and analyzed for cholic acid.

As shown in Table VI, incubation of cholic acid with a fresh suspension of cecal contents for 48 hours led to the decomposition of 95 per cent of this bile acid. No decomposition occurred either in the flask containing the heated cecal contents or in the control flask. This experiment showed conclusively that the cecal contents contained a heat-labile agent that converted cholic acid to a derivative which did not give the Gregory-Pascoe reaction.

Experiment 7. Cholic Acid Decomposition by Alcaligenes faecalis Iso-lated from Guinea Pig Cecum—It seemed probable that decomposition of cholic acid by cecal contents was due to bacterial action, since the cecum has a large bacterial flora and no enzymatic secretion of its own. Accordingly, the various aerobic bacteria in the cecum were isolated in pure culture and tested for their abilities to decompose cholic acid according to the following scheme. A 500 cc. Erlenmeyer flask containing 50

TABLE VI

Experiment 6. Decomposition of Cholic Acid in Media Containing Suspension of Cecal Contents

Each flask contained approximately 100 mg. of sodium cholate dissolved in 50 cc· of infusion broth.

Flask No.	Cecal contents added to flask	Cholic acid per 100 cc. medium		Cholic acid decomposed
		Incubated 0 hr.	Incubated 48 hrs.	
		mg.	*mg.*	*per cent*
1	0.5 cc., 1:20 dilution	167	8 4	95 0
2	0.5 " 1:20 " heated at 100° 15 min.	167	164	1.8
3	None	168	168	0

TABLE VII

Experiment 7. Cholic Acid Decomposition by Alcaligenes faecalis

	Cholic acid, mg. per cent				
	Prior to incu-bation	Hrs. after incubation			
		12	18	24	48
Flask 1. 100 cc. synthetic medium + so-dium cholate + *Alcaligenes faecalis*	210	111	37	13	0
Flask 2. Control; 100 cc. synthetic medium + sodium cholate	210	212	209	207	207

cc. of beef heart infusion broth and approximately 100 mg. of sodium cholate was inoculated with 2 cc. of an 18 hour broth culture of the desired organism, and then incubated at 38° for 96 hours. Samples of the culture, obtained prior to and after 48 and 96 hours incubation, were extracted and analyzed for cholic acid.

Only one of the eight organisms isolated lowered the cholic acid content of the medium. In the flask containing this organism, the cholic acid concentration dropped from 169 mg. per cent prior to incubation to 24 mg. per cent at the end of 48 hours. The active organism was a relatively

thin, motile, Gram-negative rod, approximately 0.5 μ in width and 2 to 6 μ in length. It occurred either singly or in long or short chains, grew well on infusion agar but poorly in infusion broth, produced neither acid nor gas from glucose, sucrose, or lactose, did not liquefy gelatin, nor form indole, and did not reduce nitrate. On the basis of these characteristics, the organism was identified tentatively as *Alcaligenes faecalis*.[1]

This organism grew fairly well in a simple synthetic medium (7), and decomposed cholic acid rapidly in this medium. In a typical experiment, such as that shown in Table VII, organisms grown on an infusion agar slant for 18 hours were washed from the slant with 5 cc. of synthetic medium and added to a 1000 cc. Erlenmeyer flask containing approximately 200 mg. of cholic acid (as the sodium salt) in 100 cc. of the medium. The flask and contents were incubated at 38° for 48 hours, with frequent shaking. Samples were withdrawn for cholic acid analysis prior to incubation and after 12, 18, 24, and 48 hours. A control flask containing only the synthetic medium and sodium cholate was treated in a similar manner.

The data shown in Table VII testify to the rapid decomposition of cholic acid by pure cultures of *Alcaligenes faecalis* obtained from the guinea pig cecum. Within 24 hours, more than 90 per cent of the cholic acid was converted into a derivative that would not give the Gregory-Pascoe reaction.

DISCUSSION

The present study provides direct experimental evidence for the catabolism of cholic acid. Some direct evidence has been supplied heretofore by the experiments of Rosenthal *et al.* (8), Kim (9), and Sasaki (10), but these experiments were preliminary and not conclusive. Indirect evidence has been presented in the studies of Bollman and Mann (6) and Berman and associates (11).

In view of our observations, the process involved in the catabolism of cholic acid in the guinea pig may be summarized as follows: The injected bile acid is removed from the blood by the liver and secreted into the small intestine through the bile. A considerable portion of the secreted material is absorbed from the small intestine. The remainder passes into the cecum where it is acted upon by the bacteria present in this organ. It seems probable that the cholic acid absorbed from the small intestine is resecreted into this organ through the well known enterohepatic circulation. Again a portion of cholic acid escapes absorption and passes into the cecum, this process probably being repeated until all of the material passes into the cecum.

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[1] We are indebted to Dr. A. G. Wedum, College of Medicine, University of Cincinnati, for assistance in this identification.

Whether all the cholic acid is catabolized in the cecum or whether another organ takes part in this process cannot be stated categorically at present. It is evident, however, that in the absence of a functionally active cecum, there is little cholic acid catabolism. Furthermore, the liver, which has been implicated indirectly in bile acid catabolism by the work of Bollman and Mann (6) does not attack cholic acid *in vitro*; our results in this respect agree with the earlier findings of Rosenthal *et al.* (8).

One may suggest that a similar catabolism of cholic acid occurs in animals other than the guinea pig. In animals like man, that do not possess a cecum comparable to that of the guinea pig, the process probably goes on in the large intestine. The fact that cholic acid can be detected in human feces (1) is proof of the presence of this acid in the large intestine. Although *Alcaligenes* and similar organisms are found in the latter organ, it remains to be proved that the strains found in the human intestine are able to catabolize cholic acid.

It should be pointed out that the conception of bacterial decomposition of the bile acids is not a new one. As early as 1886, Mylius (12) reported that cholic acid was converted to desoxycholic acid during putrefaction of bile; Wieland and Sorge (13) were unable to confirm this observation, however. Later Exner and Heyrovsky (14) showed that sodium cholate solutions could not be precipitated with lead acetate if previously incubated in cultures of *Escherichia coli*, *Eberthella typhosa*, or *Klebsiella pneumoniae*. Licht (15) confirmed this work and in addition found that cultures of *Pseudomonas aeruginosa* and *Bacillus proteus* had similar activity. Bondi and Hess (16) found that broth solutions of sodium cholate failed to give the Pettenkofer reaction after several days incubation with *Escherichia coli*. Although Jenke (17) could not confirm this work, his failure was probably due to use of concentrations of sodium cholate that actually inhibited growth.

In more recent years, a group of Japanese workers found that bacteria decomposed various bile acids, and isolated and identified some of the reaction products. Thus, Fukui (18) found that *Escherichia coli* reduced dehydrocholic to 7-oxy-3,12-diketocholanic acid, while Mori (19) ascribed a similar action to *Bacillus proteus*. Sihn (20) reported that *Escherichia coli* reduced 3,7-diketocholanic to 3,7-dihydroxycholanic acid. In each of these cases, the amount of acid converted was extremely small.

In conclusion, it may be said that experiments have been carried out in our laboratory to determine what cholic acid derivatives are produced by the action of *Alcaligenes faecalis*. This work, which will be reported shortly, has shown that a series of ketocholanic acids is formed. Experiments are now in progress to determine whether similar derivatives are formed in the guinea pig cecum.

SUMMARY

The catabolism of cholic acid in the guinea pig has been studied. When injected intravenously, cholic acid was secreted rapidly and quantitatively into the bile and thus into the intestinal tract. Within 96 hours after injection, cholic acid disappeared from the animal body, without being eliminated as such in either urine or feces. That the cecum was concerned in this disappearance was shown (1) by essentially quantitative recovery of cholic acid administered to the guinea pig with a functionally inactive cecum, and (2) by decomposition of cholic acid in the isolated cecum.

This decomposition of cholic acid in the cecum was due to the activity of a Gram-negative rod having the cultural characteristics of *Alcaligenes faecalis*. This organism, growing in a synthetic medium, caused rapid decomposition of cholic acid.

The significance of these findings in the catabolism of cholic acid in the guinea pig and other animals has been discussed.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XXI. UNIMOLECULAR FILMS AND FRACTIONS OF PROTYROSINASE ACTIVATORS FROM GRASSHOPPER EGG OIL*

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Since the lipoidal phase or centripetal layer in an extract of grasshopper eggs seems to change the contained protyrosinase into tyrosinase (1), it should be of some interest to see whether there is a specific native activator of protyrosinase. The following pages describe a vacuum sublimation study of this native oil. Some fractions thus obtained are compared on the basis of unimolecular film measurements.

Methods and Materials

Diapause eggs were ground up in 0.9 per cent NaCl solution and centrifuged. The centripetal layer was collected, dissolved in ligroin, and washed with water. The ligroin was boiled off, and 3.752 gm. of the yellow-colored oil were placed in a vacuum sublimation apparatus. Six fractions were drawn off; Fractions 1 and 2 were combined and resublimed into eight fractions, Fraction 3 into three, Fraction 4 into three, Fraction 5 into two, and Fraction 6 into two.

The sublimation procedure was supplemented by a chemical method of fractionation. The neutral oil (5.136 gm.) from diapause eggs (172 gm.) was dissolved in 100 cc. of absolute alcohol and refluxed in the presence of 1.5 gm. of potassium hydroxide. At the end of 2 hours this preparation was mixed with 200 cc. of water and was washed at 0° with three 50 cc. portions of petroleum ether in order to remove the so called unsaponifiable fraction (0.142 gm.). The saponifiable fraction was collected by adding an excess of sulfuric acid and washing with petroleum ether. The yield was 4.347 gm. of "mixed fatty acids" which were then fractionated in the sublimation apparatus.

The Pyrex glass sublimation apparatus is illustrated in Fig. 1. The outer tube was heated by an electric oven, while the inner tube or "cold finger" was cooled by circulating tap water, ice water, or acetone-solid carbon dioxide. Fractionation was begun by placing the original material inside the bottom of the outer tube. The apparatus was evacuated by a

mechanical and then by a mercury vapor pump. At the end of a fractionation period the oven was lowered away and the apparatus allowed to cool before the vacuum was broken and the inner tube raised so that the condensate on its surface could be rinsed with ligroin into a watch-glass.

Weighed portions of various fractions were dissolved in ligroin made to approximately 0.5 per cent of absolute alcohol. These solutions were used in studies of unimolecular films. The experimental technique followed that of recent publications (2–4). Monolayers of the native oil were simi-
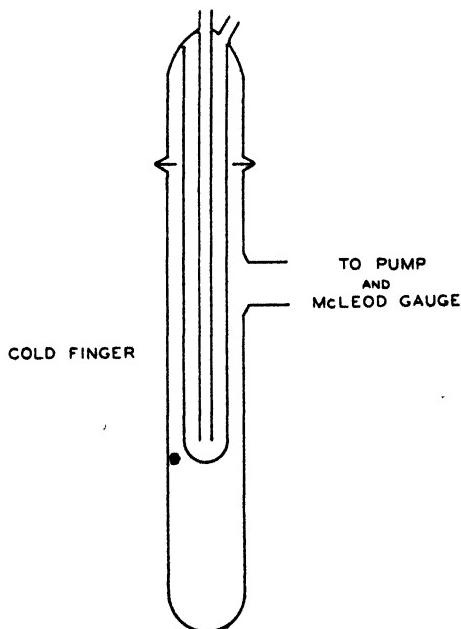
FIG. 1. Vacuum sublimation apparatus

lar on either 0.01 N HCl or Sørensen's phosphate buffer solution of pH 8.4. The experiments accordingly were performed on 0.01 N HCl subsolutions.

The neutral equivalents were found by titrating in 80 per cent alcohol with sodium hydroxide in the presence of phenolphthalein. The melting points were determined in capillary tubes within less than 2 hours after the samples had solidified.

Dispersions of the fractions were prepared by shaking in 0.10 M Sørensen's phosphate buffer solution of pH 6.8 for 10 minutes at twenty-two oscillations per second through a 6 cm. displacement. Aliquots of these dispersions were added to Warburg manometer vessels. The protyrosinase solution was prepared according to a method described earlier (5).

A typical experiment before addition of the substrate was arranged as follows: 1.0 cc. of lipid dispersion in the reaction chamber, 1.2 cc. of 0.1 M phosphate buffer in the reaction chamber, 0.5 cc. of protyrosinase solution in the reaction chamber, 0.3 cc. of 0.4 per cent tyramine hydrochloride solution in the side bulb, 0.1 cc. of 10 per cent KOH solution and filter paper in the center well. The substrate was added 30 minutes after the mixing of lipid and protyrosinase and within 20 minutes after the apparatus had been placed in the water bath at 24.9°.

The tyrosinase activity is expressed as the reciprocal of the time period in minutes for the uptake of the initial 100 microliters of oxygen.

Results

The fractionation steps, the appearance at room temperature, and the melting points are listed in Table I. The native oil yielded no perceptible deposit at room temperature, but as the temperature was increased the indicated yields were obtained. In any instance the recovery as a condensate never exceeded 70 per cent, and the higher residues had an amber color. This color and the extreme insolubility of the final residues probably show that pyrolysis had occurred. In order to test the effect of heat, the 0.070 gm. of Fraction 4 was subjected to a resublimation of $\frac{1}{2}$ hour at 150°. The weight of the condensate showed a 97 per cent recovery, and the melting point was still 45.0°.

The condensates are the fractions which are to be compared. Force-area diagrams of monomolecular films are given in Fig. 2 for the native oil and five of the fractions. These are not condensed films, because talc particles will glide freely about the surface. Plots of the product of force and area against the force, moreover, show that the films do not behave as though they were gaseous (6). The Fraction 5 film must be extremely fluid, because its viscosity proved to be insignificantly different from that of the subsolution. The general form of all the curves resembles that of a liquid expanded film above its critical temperature (6). The latter characteristic may account for the fact that these films on barium chloride subsolutions adhere to chromium plates on the down trip only to come off on the up trip (7).

The physical homogeneity of a monolayer can be judged by dark-field, ultramicroscopic examination (8) as to the absence of microscopic droplets or lenses of the film-forming substance. Lenses began to appear at the pressures of collapse in the monolayers (Fig. 2). Since the lens points (F_x) coincide with the kinks, the pressure of collapse is evidently that at which a change from two to three dimensions occurs. It thus seems that through the expanded portions the films are homogeneous.

Additional information on homogeneity (2) is given by the values of the

surface potentials. The ΔV is defined as the difference between the contact potential when the film is present and when the film is absent (6). The surface potentials of Fraction 18 and the native oil are certainly greater than those of Fraction 5 (Fig. 2). During the course of time, the surface potential is steady at areas within the expanded portions of the compression curves. Therefore, the monolayers at these areas have no patches of vary-

TABLE I

Fractionation of Native Oil

Sublimation				Resublimation				
Fraction No.	Yield	Oven temperature	Appearance at room temperature	Fraction No.	Yield	Oven temperature	Appearance at room temperature	M.p.
	gm.	°C.			gm.	°C.		°C.
1	0.185	90	Colorless solid	1	0.004	68	Colorless solid	36.2
2	0.780	122	" "	2	0.017	72	" "	36.9
			trace of pale	3	0.066	79	" "	43.9
			yellow oil	4	0.070	83	" "	45.0
				5	0.100	92	" "	45.0
				6	0.112	109	" "	40.3
				7	0.125	123	" oil with granules	27.8
				8	0.083	147	Colorless oil	28.8
3	0.739	170	Pale yellow oil	9	0.068	117	" "	25.5
				10	0.225	162	Pale yellow oil	28.0
				11	0 047	220	Yellow oil	9.2
4	0.204	178	Yellow oil, needle-shaped crystals	12	0.038	123	Colorless oil	25.3
				13	0.025	155	" solid	38.1
				14	0.024	217	" "	50.3
5	0.269	264	Yellow oil	15	0.028	135	" "	30.7
				16	0.063	231	Bright yellow oil	15.1
6	0.350	328	" "	17	0.040	168	Colorless oil	25.5
				18	0.219	316	Bright yellow oil	−5.5

ing nature. At larger areas, where the pressure is similar to that of a surface vapor pressure (6), the films are not homogeneous. Since the surface potentials vary with time and with the position of the probe electrode, islands of liquid and gas evidently occur in the low pressure films. According to both the ultramicroscope and the surface potentials, it seems that the films consist of a single phase throughout the expanded portions.

An equation relating the surface potential to the concentration of molecules in a film, as ordinarily used, is $\Delta V = 4\pi\eta\mu$ where μ is related to the standard dipole moment (6). Although the meaning of this ratio is
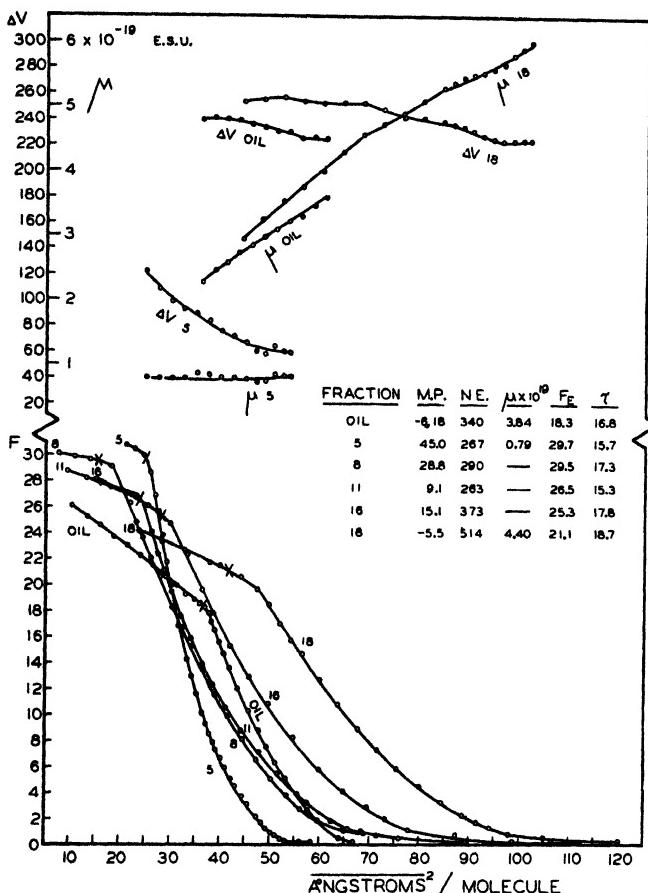
FIG. 2. Surface pressures, potentials, and μ values for the native oil and some of its fractions. F = dynes per cm., ΔV = millivolts, $M.P.$ = melting points, $N.E.$ = neutral equivalents (molecular weight), F_E = lens point or spreading pressures, τ = thickness in Å. calculated from density 0.90, temperature = 27.5–30.0°, Δt = 1 minute.

limited by a certain lack of knowledge (2), μ is useful for comparative purposes. As the area of the native oil and Fraction 18 films is reduced, their μ values decrease in a similar way. On the other hand these values remain constant when the Fraction 5 film is compressed. The decreasing μ values

of the oil and Fraction 18 may mean that the tilt of the dipoles to the surface is decreasing. In the Fraction 5 monolayer a change in area does not seem to change the tilt of the dipoles; rather than being tilted up the molecules of this film seem to remain flat.

The fractionation of the mixed fatty acids was carried out according to
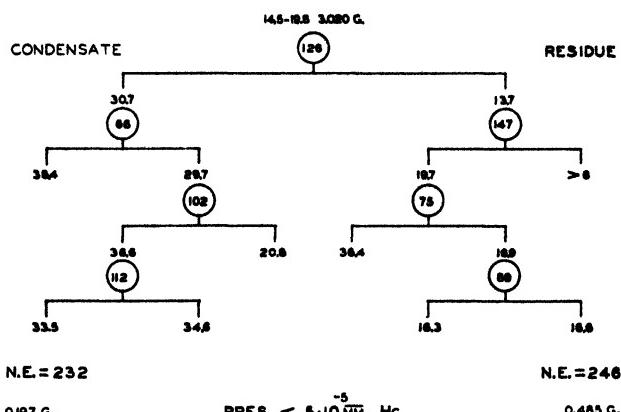
FIG. 3. Fractionation of the mixed fatty acids. Oven temperatures are given inside the circles. The melting points are indicated in the fractionation "tree."

TABLE II

Comparison of the Fractions from Mixed Fatty Acids with Saturated Fatty Acids at 25°

Acid	M.p.	Mol. wt.	Limiting area	Surface vapor pressure	$\mu \times 10^{18}$	Compressibility
	°C.		$\overset{-2}{A}$.*per molecule*	*dynes per cm.*	*electrostatic units*	$-\kappa$
Tridecylic, C_{13}	40.5	214	47.0	0.30		>0.026–0.06
Myristic, C_{14}	54.0	228	46.4	0.19	1.8	>0.04 –0.07
Pentadecylic, C_{15}	52.1	242	45.6	0.11	1.8	0.035–0.045
Palmitic, C_{16}	63.0	256	26.65	0.04	2.3	0.008–0.010
Condensate, C_{13} (?)	16.6	232	55.5	0.29	1.76	0.04 –0.08
Residue, C_{14} (?)	33.5	246	49.2	0.22	1.60	0.04 –0.08

the schedule listed in Fig. 3. It was believed that a condensate and a residue, containing different fatty acids, could be isolated by resublimation. This procedure was continued until there was no great difference between the melting points of a condensate and a residue (Fig. 3). Monolayers prepared from these fractions were examined on a "vertical pull" film balance (3). It seems that saturated fatty acids were obtained; on a subsolution of

0.01 per cent $KMnO_4$ and 0.01 N H_2SO_4 there is no change in film character-istics such as is to be expected with monolayers of unsaturated fatty acids (9). The melting points, neutral equivalents, limiting areas, surface vapor pressures, μ values, and compressibilities (10) are compared (Table II) with constants of four saturated fatty acids.

The inset of Fig. 4 exhibits the decrease in protyrosinase or the increase in tyrosinase wrought by the presence of increased concentrations of the condensate and the residue from the mixed fatty acids. The velocity of the enzymic reaction varies directly with the amount of activated proty-
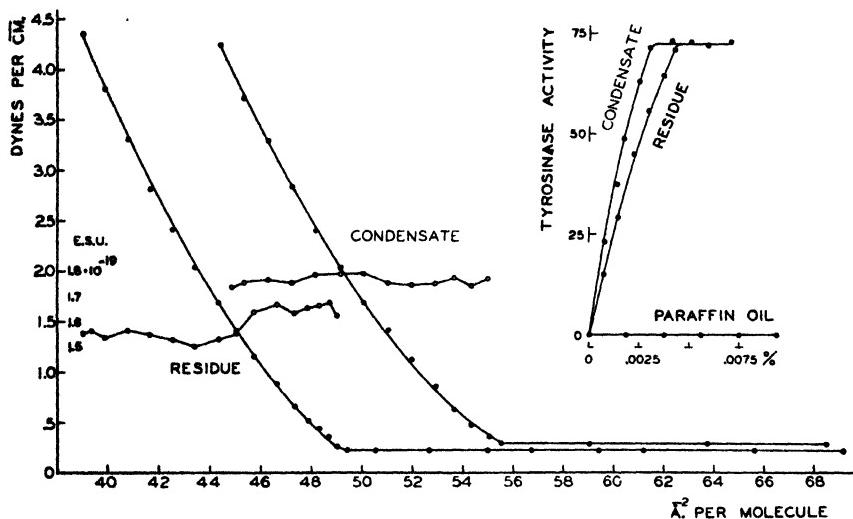
FIG. 4. Surface pressures and μ values for two fractions from the mixed fatty acids. Temperature = 23.5–24.5°, Δt = 1 minute. Inset, the effect of these fractions and petrolatum on protyrosinase. Temperature = 24.9°.

rosinase and thus may be used as a measure of the amount of tyrosinase (11). The other fractions from the native oil were also found to be capable of activating protyrosinase. The results of these experiments seem to show that the native oil or its various fractions will change protyrosinase into tyrosinase. On the other hand dispersed paraffin oil seems incapable of activating protyrosinase (Fig. 4, inset).

DISCUSSION

When one compares sublimation temperatures with one another, a con-servative estimate would place at least two separate compounds among the present fractions. Certain differences in the monolayers, the variety of melting points, and a number of neutral equivalents also make it appear

probable that while a particular compound is present in one group of fractions it is absent in others. According to this evidence, one may assume that the oil from eggs of a grasshopper is of a "mixed constitution" (12).

Since all the fractions will activate protyrosinase, it seems reasonable to conclude that no specific activator is contained in this native oil. These results agree with those of other studies in which various polar-non-polar compounds were shown to be capable of activating protyrosinase (11). But an' aliphatic hydrocarbon oil, petrolatum, is not an activator (13). It seems as though lipoidal compounds are able to activate protyrosinase by virtue of the common possession of similar chemical groups. Since the paraffin oil has no polar groups and does not activate protyrosinase, the atoms of the activating groups probably compose the polar groups of interest to surface chemistry.

The protyrosinase entity at present remains purely conceptual, but some explanations of its activation seem to be in order. These ideas are concerned with the various activators which may be classified as to their generally accepted behavior. Certain periods of time exposure and degree of treatment with heat, with various concentrations of acetone, urea, or urethane, and the removal of electrolytes are known to affect the nature of proteins. Because similar treatments have been also found to activate protyrosinase, it seems by analogy that the activation may involve a particular structural change of the protyrosinase molecule. Perhaps, similar changes are brought about by the polar groups of the lipid activators. The chemical configuration of the tyrosinase molecule may then be such as to permit the catalytic oxidation of substrates via electron exchange involving the active center or group, which seems to contain the metallic element, copper (14, 15).

SUMMARY

1. Eighteen fractions were obtained by vacuum sublimation from the oil of grasshopper (*Melanoplus differentialis*) eggs. Two fractions were obtained by continued resublimation of the "mixed fatty acids."

2. Each one of the twenty fractions activates protyrosinase.

3. It is concluded that the oil is of a mixed constitution and also that it contains no specific activator of protyrosinase.

4. The activity of the dispersed fractions is discussed in terms of the effect of polar groups on protyrosinase.

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LETTERS TO THE EDITORS

THE EFFECT OF TESTOSTERONE PROPIONATE ON THE ARGINASE CONTENT OF THE LIVER, KIDNEY, AND INTESTINE*

Sirs:

It has been demonstrated in this laboratory that androgens produce a positive nitrogen balance in castrate dogs.[1] This observation has been confirmed in humans.[2] It seemed worth while to determine what becomes of the retained nitrogen. Therefore, as one means to this end, a study of the various enzymes concerned with growth processes has been initiated. The results obtained with respect to the effect of testosterone propionate on arginase activity warrant a preliminary report.

The rats were litter mate pairs, which were castrated at about 70 gm. of body weight. Injection of 5 mg. per day of testosterone propionate[3] was begun about 5 days after castration. On the 16th day, a pellet of testosterone propionate (20± mg.) was implanted in each experimental animal and the injections stopped. 2 weeks later the arginase content of the intestine, liver, and kidney was determined by the method of Light-body[4] with minor modifications. The results in the accompanying table show marked increases in the arginase content of the three tissues studied with the greatest effect in the kidney. Similar increases have been obtained on normal and castrate mice treated for 1 month by pellet of testosterone propionate.

These results are in agreement with our assumption that the retained nitrogen as a result of androgen administration is used for growth processes. Edlbacher and associates (*cf.* Baldwin[5]) have demonstrated that growing

[1] Kochakian, C. D., and Murlin, J. R., *J. Nutrition*, 10, 437 (1935); *Am. J. Physiol.*, 117, 642 (1936). Kochakian, C. D., *Endocrinology*, 21, 750 (1937).

[2] Kenyon, A. T., Sandiford, I., Bryan, A. H., Knowlton, K., and Koch, F. C., *Endocrinology*, 23, 135 (1938). Albright, F., Parson, W., and Bloomberg, E., *J. Clin. Endocrinology*, 1, 375 (1941). Basset, S. H., Kochakian, C. D., and Friedman, H. A., unpublished.

[3] The testosterone propionate was supplied under the trade name "perandren" by Ciba Pharmaceutical Products, Inc., through the kindness of Dr. E. Oppenheimer.

[4] Lightbody, H. D., *J. Biol. Chem.*, 124, 169 (1938).

[5] Baldwin, E., *Biol. Rev.*, 11, 247 (1936).

Treatment		Micromoles urea per gm. per 100 gm. body weight × 10⁻⁸					
	Testosterone propionate absorbed from pellet*	Liver		Kidney		Intestine	
days	mg.		per cent		per cent		per cent
Control		56.6	+55	2.17	+14	0.56	+51
33	4.3	87.5		2.46		0.84	
Control		30.0	+50	1.09	+206	0.47	+23
36	4.5	44.9		3.33		0.58	
Control		28.3	+53	1.23	+380	0.45	+225
39	3.8	43.4		5.93		1.44	
Control		12.2	+155	1.25	+104	0.57	+39
41	4.5	30.1		2.55		0.79	

* These values are for the amount of testosterone propionate absorbed from the pellets implanted on the 16th day of treatment. Up to the time of pellet implantation, each experimental animal received subcutaneously 5 mg. per day of testosterone propionate in oil.

tissues as a whole possess high arginase activity. They have expressed the view that the increased arginase is not for the formation of urea, but for the synthesis of protein components of nucleoplasmic material. It might be assumed, therefore, that androgens have a similar effect, since they cause a decrease in urea elimination and an increase in arginase activity.

Experiments with d-amino oxidase show similar increases in the liver and kidney.

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PREPARATION OF TRYPTOPHANE-CONTAINING ACID HYDROLYSATES OF PROTEINS SUITABLE FOR INTRAVENOUS ADMINISTRATION*

Sirs:

The problem of the parenteral administration of nitrogen derived from protein sources is of importance particularly in circumstances in which hypoproteinemia may develop. It then becomes necessary to supply protein, or protein precursors, to an individual who, because of either his clinical condition, the loss of voluntary action, or other reasons, is unable to obtain or utilize orally administered protein. It has been established that experimental animals and humans utilize parenterally administered nitrogen for maintenance of positive nitrogen balance and for plasma protein regeneration. The sources of nitrogen commonly employed have generally been products prepared from proteins by acid hydrolysis or by enzymatic digestion.

The acid hydrolysis procedure is ideal except for the decisive difficulty that under the usual conditions employed extensive destruction of tryptophane results. Since this amino acid is essential for production of nitrogen retention and plasma protein synthesis, tryptophane must be added to acid hydrolysates. The cost of tryptophane makes this approach to the clinical problem impractical. Enzymatic digests of casein have proved quite satisfactory for parenteral administration; however, the enzymatic process, while preserving tryptophane, has undesirable features: (1) the time required for protein digestion, and (2) the use of crude enzyme sources for large scale preparations. The first point is not completely objectionable; the second is minimized by employing purified enzymes. However, when large quantities of digests are needed, the cost of better enzyme preparations becomes of importance. Ground, fresh pancreas has been employed for the commercial preparation of enzymatic digests. While satisfactory products have been obtained,[1] there always exists the possible contamination of the enzyme source with a variety of tissue substances which might appear in the final preparation.

A more satisfactory solution of the problem appeared to be the development of conditions of acid protein hydrolysis which would (1) hydrolyze the protein to smaller fragments that produce no undesirable reactions when rapidly injected intravenously, and (2) cause minimal destruction

[1] Elman, R., *Ann. Surg.*, **112**, 594 (1940).

of the amino acids of the protein, with particular reference to tryptophane. These conditions have now been established.

Hydrolysis of casein or of pumpkin seed globulin with 2.6 N sulfuric acid for 6 hours liberates 60 per cent (for casein) and 55 per cent (for pumpkin seed globulin) of the total amino nitrogen liberated by the usual conditions of protein hydrolysis (8 N sulfuric acid, 24 hours boiling). Tryptophane analyses[2] of these hydrolysates indicate that 85 per cent of the tryptophane present in casein is not destroyed. 65 per cent of the total tryptophane of pumpkin seed globulin is found in similarly prepared hydrolysates. Sulfuric acid is removed with baryta, the barium sulfate filtered and washed, and the filtrates and washings decolorized with norit and concentrated to dryness *in vacuo*. The final products are light colored powders, very soluble in water, and produce no undesirable reactions in dogs when administered rapidly in solution by vein. The similarity of the amino acid composition of these preparations to that of the starting proteins is indicated from two types of comparative investigations, (1) rat growth experiments and (2) nitrogen balance studies in dogs. Plasma protein regeneration studies are in progress.

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